University of Alberta

# The Biological significance of loss of SHP1 in the pathogenesis of ALK+ anaplastic large cell lymphoma

by

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## Abstract

SHP1, a tyrosine phosphatase, is an important negative regulator in various cellular signaling pathways including that of JAK/STAT. Most cases of ALK+ ALCL tumors, which are characterized by the expression of the oncogenic fusion gene protein NPM-ALK and constitutively activated JAK3/STAT3, are negative for SHP1. To assess the biological significance of the loss of SHP1 in ALK+ ALCL, we restored SHP1 expression by: 1) 5-AZA-2'-deoxycytidine, an inhibitor of DNA methyltransferase, and 2) SHP1 gene transfection in ALCL cell lines confirmed to be SHP1 negative. We found that the restoration of SHP1 in ALCL cell lines resulted in a decrease of JAK3/STAT3 signaling activity and decrease in the protein levels of JAK3 and NPM-ALK, associated with cell cycle arrest. Further study showed that two mechanisms are involved in: (1) dephosphorylation of the active form of JAK3, the physiologic activator of STAT3, (2) instabilization of the JAK3 and NPM-ALK proteins by upregulating their degradation through the proteasome pathway. These conclusions were further supported by downregulating SHP1 in the SHP1+/ALK+ ALCL cell line by using SiRNA. Immunoprecipitation results revealed that SHP1, JAK3 and NPM-ALK bind together physically, which likely provides the basis of the functional interaction among these proteins. In summary, loss of SHP1 carries a significant role in the pathogenesis of ALCL and restoration of SHP1 may be a useful therapeutical approach.

*To*

*My loving and supportive husband Jinsuo and our son John Xiuqi, who accompanies with me in Canada and always tries to help me to complete this study.*

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# List of Abbreviation

## A

AG490: a protein tyrosine kinase inhibitor that specific for JAK

AKT: The other name of protein kinase B (PKB), a serine/threonine protein kinase

ALL: acute lymphocytic leukemia

ALCL: anaplastic large cell lymphoma

ALK: anaplastic lymphoma kinase

ALK+ ALCL: ALK positive, or, ALK expressing ALCL

ALK- ALCL: ALK negative, or, no ALK expressing ALCL

5-AZA: 5-aza-2'-deoxycytidine

AML: acute myeloid leukemia

## B

Bcr/Abl: oncogenic tyrosine kinase that resultes from chromosome t(9;22)(q34;q11) translocation, and is specific for chronic myelogenous leukaemia

Bcl-2: B-cell leukemia/lymphoma 2

Bcl-xL: Bcl-x long form, a protein of Bcl-2 family

## C

CD30: cell surface marker expressed by some lymphocytes. CD30 is a tumor necrosis factor receptor. It is associated with anaplastic large cell

Lymphoma

CML: chronic myelogenous leukaemia

CpG island: a short stretch of DNA in which the frequency of the CG sequence is higher than other regions. The "p" simply indicates that "C" and "G" are connected by a phosphodiester bond. CpG islands are often located adjacent to the promoters.

CSF: colony-stimulating factor

Cyclin D3: a positive cell cycle regulator

## D

DMSO: dimethyl sulfoxide

DNMT1: DNA methyltransferase 1

## E

Epo: erythropoietin

EpoR: receptor of Epo

EGF: epithelial growth factor

EcoR1: a specific restriction endonuclease

EcoRV: a specific restriction endonuclease

EDTA: Ethylenediaminetetraacetic acid

## F

FBS: fetal bovine serum

## G

GFP: green fluorescent protein

EGFP: enhanced green fluorescent protein

GP293 cells: an embryonic kidney cell line

## H

HTB 26 cells: a breast cancer cell line

## I

INF: interferon

IRS-1: Insulin receptor substrate-1

IL2: interleukin-2

IL-2R: receptor of IL-2

## J

JAKs: janus kinases

pJAK3: phospho-JAK3, or, active JAK3

## K

K562 cells: a specialized cell line of human erythroleukemia cells

c-Kit: stem cell factor receptor, or, CD117

## M

MAPK: mitogen activated protein kinase

Mcl-1: induced myeloid leukemia cell differentiation protein Mcl-1

MDS: myelodysplastic syndrome

MEN2A: multiple endocrine neoplasia 2A

MG132: a proteasome inhibitor

## N

NHL: non-Hodgkin lymphoma

NPM: nucleophosmin

NPM-ALK: a fusion protein resulted from chromosome 2p23(ALK) and chromosome 5q35 (NPM) translocation

## P

PARP: poly (ADP-ribose) polymerase

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PI3-k: phosphatidylinositol 3-kinase

PIAS: protein inhibitor of activated STAT

p-Tyrosine: phospho-tyrosine

PLC-γ : Phospholipase Cγ

PTPs: protein phosphatases

PTKs: protein tyrosine kinases

$P15^{INAK4B}$ : p15, inhibits CDK4.

P15: cyclin-dependent kinase inhibitor 2B

RT-PCR: reverse transcription PCR

## R

RTK: receptor tyrosine kinase

RAS: proteins belong to the superfamily of monomeric GTPases

RAS/MAPK pathway: RAS proteins belong to the superfamily of monomeric GTPases, which are involved in receptor-mediated signal transduction pathways. The major downstream effecter of RAS is mitogen-activated protein kinase (MAPK)

Ret: a receptor tyrosine kinase

## S

SHC: The phosphotyrosine (pTyr) adapter

SH2: the Src homology domain -2

SHP: SH2-containing phosphatase

SHP1+: SHP1 positive, or, SHP1 expressing

SHP1-: SHP1 negative, or, no SHP1 expressing

SiRNA: small interfering RNA

SOCS: Suppressors of cytokine signalling

Src: a protein kinase obtained from Src sarcoma virus originally

STATs: signal transducers and activators of transcription

pSTAT3: the phospho-STAT3, or, active STAT3

## U

U266 cells: a myeloma cell line

## X

Xho 1: a restriction endonuclease

# Chapter 1: Introduction

## Section 1.1 Introduction to anaplastic large cell lymphoma

The first description of ALCL by Stein *et al* was published in 1985.[1] ALCL was initially recognized as a subtype of T-cell/null-cell Non Hodgkin lymphoma characterized by an anaplastic tumor cell cytology, sinusoidal infiltration pattern, and the expression of CD30. Benharroch D *et al*[2] have described the most characteristic cells in ALCL as large lymphoma cells containing an eccentric horseshoe or kidney-shaped nucleus, visible but not prominent nucleoli, and an eosinophilic Golgi body. Currently, ALCL is well-recognized as a distinct clinicopathological entity in the WHO (World Health Organism) classification system, and these tumors account for 5% of all human NHL and 30-40% of pediatric NHL. [3,4]

The chromosome alteration in ALCL was first reported in the late 1980s, [5] and the t(2:5) translocation was identified and cloned in 1994 by Morris and his colleagues.[6] Approximately half of ALCL have t(2;5) chromosomal translocations and 80% of these translocations are the t(2;5)(p23;q35) translocation, which results in the expression of an oncogenic fusion protein NPM-ALK.[2,6-9] As described later in this chapter, NPM-ALK is a tyrosine kinase that is constitutively activated and has extensive interaction with multiple signaling proteins, such as

PLC-γ, STAT3 and JAK3, which have extensive effects on a wide range of cellular biological behaviors, such as proliferation and survival.

Not all tumors classified as ALCLs have the t(2;5)(p23;q35) chromosomal translocation and the expression of the fusion protein NPM-ALK. Thus, ALCLs are further divided into ALK+ ALCL and ALK- ALCL. The ALK+ ALCL has different clinical characteristics and prognosis from the ALK- ALCL. Generally, ALK+ ALCL always affects young male patients and has a better clinical outcome.[8,10] This thesis focuses only on ALK+ ALCL. The pathogenesis of ALK+ ALCL is not clear, but NPM-ALK is strongly suspected as the main driving force for the pathogenesis of ALK+ ALCL.[11-15]

## Section 1.2 Overview of NPM-ALK

In 1988, the t(2;5)(p23;q35) chromosomal translocation was indented by Fischer et al[6] in the Karpas 299 cell line derived from an ALCL patient. This chromosomal translocation was soon confirmed in additional ALCL cell lines and tumors.[16-19] In 1994, the breakpoint of t(2;5) was cloned by Morris et al, and a monoclonal antibody against the ALK1 protein was developed.[20] Using the anti-ALK1 antibody, more ALK fusion proteins other than NPM-ALK were recognized.[2,21,22] All the ALK fusion proteins were the results of chromosomal translocations involving 2p23(ALK) and one of its fusion partners, which could drive the dimerization of

ALK and result in the constitutive activation of the ALK tyrosine kinase.[23-26] The t(2;5)(p23;q35) chromosomal translocation, which causes fusion of the ALK and NPM genes, leads to the expression of a 80-kD functional fusion protein NPM-ALK containing the N-terminal portion of NPM (amino acids 1-117)[6,27] linked to the entire cytoplasmic domain of the neural-associated receptor tyrosine kinase ALK.[28,29]

NPM (nucleophosmin), which is located at 5q35, is ubiquitously expressed in all cell types. Normally, NPM functions as a shuttler for ribonucleoproteins between the nucleus and cytoplasm. Structurally, ALK belongs to the insulin receptor tyrosine kinase (RTK) family. Located at 2p23, it is strictly expressed in the nervous system.[6] *In vitro* studies showed that ALK is responsible for the development and function of the nervous system.[20, 28,29] The novel fusion protein NPM-ALK is oncogenic and possesses transforming ability[11,12,13] and can induce large cell lymphomas *in vivo*.[14,15,30] The NPM portion of NPM-ALK provides the oligomerizing motif that drives the homodimerization of NPM-ALK as well as heterodimerization between NPM-ALK and wild-type NPM.[11] The dimerized NPM-ALK is autophosphorylated, constitutively activated at the tyrosine kinase, which activates a number of downstream effectors to mediate its roles on cell proliferation, survival, and transformation.

It has been reported that NPM-ALK modulates cell mitogenesis through the

RAS/MAPK and PLC-γ pathways. As adaptors for RAS/MAPK pathway, SHC and IRS-1 were found to bind with NPM-ALK physically[11,31], suggesting the roles of NPM-ALK in the activation of the RAS/MAPK pathway. However, the exact roles of SHC and IRS-1 in the NPM-ALK mediated oncogenesis were unknown because the site mutation studies showed that the lack of SHC and IRS-1 binding to NPM-ALK does not abrogate the transforming ability of NPM-ALK.[31] These results also suggest the possibility that some mechanisms other than the RAS/MAPK pathway exist in the mitogenesis mediated by NPM-ALK.

As an important pathway in mitogenesis, PLC-γ was identified to bind with NPM-ALK.[13] Impeding the interaction of NPM-ALK and PLC-γ by site mutation, NPM-ALK loses its transforming ability.[12] It is also been demonstrated that NPM-ALK possesses anti-apoptotic functions, which are mediated by the PI3-kinase/AKT pathway.[13,32] NPM-ALK interacts with PI3-Kinase/AKT through the SH2 domain in its C-terminal. The NPM-ALK transformed Ba/F3 cells express activated PI3-Kinase activity and inhibitor of the PI3-K abrogates the transforming ability of NPM-ALK.

Recently, it has been reported that STAT3 is important in ALK+ ALCL. STAT3 has been shown to be constitutively activated in ALK+ ALCL cell lines and NPM-ALK transformed cells.[30,33-35] Amin *et al*[36] reported that transfection of dominant

negative STAT3 to ALK+ ALCL cells induced cell cycle arrest and apoptosis. Charile et al [37] reported that neutralizing STAT3 activity by injecting antisense oligonucleotides of STAT3 could inhibit the tumor growth in mice induced by NPM-ALK. The tumorigenesis capacity of NPM-ALK depends on the levels of STAT3 expression. STAT3 is required for the tumorigenesis and tumor maintenance in tumors induced by NPM-ALK, and the NPM-ALK transformed cells are not viable without STAT3 expression.[37]

In summary, the tumorigenesis of NPM-ALK+ ALCL is complicated. However, it is evident that NPM-ALK mediates its oncogenic activity via deregulating multiple signaling pathways, and STAT3 is particularly important in this process.

## Section 1.3 Overview of the STATs signaling

STAT is a family of cytoplasmic proteins that translate the cell surface receptor-generated signals into intra-cellular signals that typically modulate gene expression. There are seven members belonging to the STAT family, which are STAT1-6, including STAT5a and STAT5b, which are encoded by two different genes. In addition, different isoforms of several STATs have been identified.[38] Normally, STAT proteins are latent in the cytoplasm until they are activated by extracellular signaling proteins (mainly cytokines and growth factors) that bind to specific cell-surface receptors.[39-41] These extracellular signaling proteins can activate various tyrosine kinases in the cell that phosphorylate STAT proteins. The

activated STAT proteins accumulate in the nucleus to drive gene transcription.[38]

In the hematopoietic system, the STAT proteins play key roles in immune response, hematopoietic cell growth and differentiation in all lineages.[42] The functions of STAT proteins are under tight regulation and any disturbance in the regulation (both activation and inhibition) leads to constitutive activation of the STATs activity, which is tumorigenic.[43]

## Section 1.4 NPM-ALK and constitutive activation of STAT3

Of the seven currently known STAT family members, STAT3, STAT5, and to a lesser extent, STAT1 are frequently constitutively activated in malignant cells.[44] Transient STAT3 activation is found in hematopoietic cell maturation.[40,45] Constitutive STAT3 activation is found in a number of primary human tumors, including ALCL.[30,33-35]

In addition to cytokines and growth factors, the STAT proteins can also be activated by some proteins that possess tyrosine kinase activity, such as Src and BCR/ABL1.[47-49] The existence of the constitutively activated NPM-ALK tyrosine kinase has been demonstrated to be the key reason for the dysregulation of STAT3 in ALK+ ALCL,[33,36] and inhibition of ALK enzymatic activity suppresses STAT3 activation.[50] The interaction between NPM-ALK and STAT3 appears to

play important roles in mediating the oncogenic effects of NPM-ALK.

In addition to the NPM-ALK tyrosine kinase, it has also been noted that some other factors contribute to the dysregulation of STAT3 activity in ALK+ ALCL. JAK3, as the physiologic activator of STAT3, has been reported to contribute to the activation of STAT3 in ALK+ ALCL.[50,51] Loss of the negative regulators, such as SHP1 and SOCS, has been suggested to be the other reason for the constitutive JAK3/STAT3 activation in ALK+ ALCL.[34,52]

## Section 1.5 Negative regulators of STAT3 signaling

There are three families of proteins: 1) the protein tyrosine phosphatases (PTPs), 2) the protein inhibitors of activated STATs (PIAS), and 3) the suppressors of cytokine signaling (SOCS), that have been shown to inhibit STAT signal transduction.

The SH2 domain-containing tyrosine phosphatases (SHP) have been well studied and shown to be important in many cytokine signaling pathways including that of the JAK/STAT.[53,54] There are two members of the SHP family in mammals, SHP1 and SHP2. In contrast to the ubiquitous expression of SHP2, SHP1 is primarily expressed in hematopoietic cells and it has effects on hematopoietic cell growth, differentiation, cell cycle control and immune response.

The biological roles of SHP1 were initially studied in mice that carry homozygous mutation in SHP1.[55,56] Mice that express either no SHP1 or a catalytic defective SHP1 display multiple hematopoietic abnormalities as exemplified by an enormous expansion and accumulation of the myeloid/monocytic cells, which are associated with patchy dermatitis, and thus labeled as 'motheaten'.[57,58] Mice that lack the catalytic domain of SHP1 manifest premature thymic involution, over expansion of the normal minor B-1 cell (CD5+) population, and an increased propensity of developing CD5+ lymphomas.[59,60] The findings based on the study of motheaten mice clearly demonstrate that SHP1 is critical for hematopoietic cellular development and homeostasis. Lack of SHP1 can result in profound immunological dysfunction, dysregulated hematopoiesis, and increased susceptiblity to developing hematopoietic malignances.[61]

SHP1 is a 69kD non-transmembrane protein phosphatase that contains two consecutive SH2 domains. It suppresses signals mediated by a variety of cytokine receptors and receptor tyrosine kinases, including the receptors for Epo, IL-3, IFN-α, CSF-1, etc.[62-64] SHP1 has also been shown to modulate JAK/STAT signaling by binding to active JAK kinase through its SH2 domain and negatively regulates the activation-evoking signaling cascades conducted through JAK/STAT signaling pathway.[62,64,65]

The activation of JAK/STAT can also be negatively regulated by the SOCS and PIAS proteins. As downstream effectors of STAT, the activity of SOCS proteins is mediated by STAT directly. The SOCS proteins, after induced by STAT activation, block the continued signaling of STATs by binding to the docking site of the active receptors and/or JAKs.[66-68] Different from SHP1 and SOCS, the PIAS proteins inhibit JAK/STAT signaling in the nucleus. The PIAS proteins bind to active STAT dimmers directly and inhibit their DNA binding ability and thus suppress gene transcription promoted by the activated STATs.[69,70]

Because SHP1 is constitutively expressed in the cytoplasm and activated immediately after the receptors are bound by their relevant ligands, SHP1 builds up the first and rapid-reacting barrier for inhibiting the cytokine signal transduction in cytoplasm. PIAS, as the second barrier for signal transduction, reduces the signal pulse in the nucleus. SOCS proteins, as the products of JAK/STAT signaling activation, dampen the signaling further. Because the expression of SOCS and PIAS proteins are dependent on the activation of JAK/STAT signaling, their modulation is slower than that of SHP1.[71]

## Section 1-6 SHP1, the cancer suppressor

Accumulated data indicate that SHP1 has cancer suppressor function.[72] As an SH2 domain containing cytoplasmic PTP, SHP1 negatively regulates multiple

hematopoietic signaling pathways by controlling the tyrosine phosphorylation mediated by PTKs,[53,54] and thereby maintaining hematopoietic homeostasis.[73] It is reported that loss of SHP1 associated with gene methylation is common in hematopoietic malignances.[74-77] Restoration of SHP1 in some cancer cells can inhibit cell proliferation.[76,78] Likewise, K562 cells transfected with SHP1 exhibited remarkable differences in growth pattern and morphology. Comparing to the cells transfected with empty vector, K562 cells transfected with SHP1 show a slower growth pattern and lower clone formation ability in soft agarose gels.[79] These data are consistent with the growth inhibitory effect of SHP1 in human malignances.

In some human malignances, dysregulated tyrosine kinase activity result from gene mutations and the expression oncogenic tyrosine kinase, such as the BCR/ABL1 in CML, Ret in MEN2A thyroid carcinoma, as well as NPM-ALK in the ALK-expressing ALCL. With the expression of these oncogenic tyrosine kinases, the normal controlled signaling pathways are constitutively activated and they lead to abnormal cell proliferation and transformation. It is reported that introduction of SHP1 can suppress the tyrosine kinase activity of some onco-proteins and inhibit their transforming ability.[80,81]

Taken together, these results strongly indicate that SHP1 is a negative regulator of abnormal kinase activity involved in the cell transformation. Therefore, loss of

SHP1 should contribute to the tumorigenesis associated with dysregulated tyrosine kinase activity.

## Section 1-7 Aberrant methylation of gene promoter and the loss of SHP1 expression

DNA methylation is a normal function for cells to prevent the over-transcription of genes. It plays an important role in tissue- and stage- specific regulation, genomic imprinting, and X-chromosome inactivation, and has been shown to be essential for normal mammalian development.[82-84] However, recent data suggest that aberrant hypermethylation of the CpG islands in the promoter regions is a common characteristic of cancer cells.[85,86] The abnormal promoter hypermethylation takes part in the inactivation of virtually all pathways involved in the neoplastic process, including DNA repair, cell cycle regulation, and apoptosis.[87] It is reported that more than half of the tumor-suppressor genes can be inactivated in association with promoter hypermethylation.[88,89] These data reveal the roles of promoter hypermethylation in the pathogenesis of cancers.[90]

In hematologic malignances, aberrant DNA hypermethylation is believed to have relevance to leukemogenesis.[91] It is reported that hypermethylation of the $p15^{INAK4B}$ tumor suppressor gene is associated with its inactivation in more than half of the patients with acute lymphoblastic leukemia (ALL) and acute

myelogenous leukemia (AML).[92,93] Moreover, Quesnel *et al*[94] found that hypermethylation of p15$^{INAK4B}$ is concomitant with the disease progression in myelodysplastic syndrome (MDS).

The perturbation of the cytokine-signaling pathway plays an important role in hematopoietic tumors.[43,95] As an important negative regulator for cytokine signal conduction in hematopoietic cells, SHP1 was often found silent due to aberrant promoter hypermethylation in hematopoietic malignances.[74,75,96,97] Reddy *et al*[97] demonstrated that there is a 100% concordance between the hypermethylation and the loss of SHP1 in their tested lymphoid/leukemia samples. Further, two study groups found that 5-AZA, a DNA methltransferase inhibitor, could induce SHP1 expression and downregulation of cell proliferation in hematopoietic malignances.[80,97] NPM-ALK+ ALCL, as a hematopoietic malignancy with dysregulated cytokine signaling provides an ideal model to study the roles played by SHP1 in the tumorigenesis mediated by oncogenic tyrosine kinases.

## Section 1-8 Summary of reviews and justification of the hypothesis

It is well demonstrated that NPM-ALK is the driving force for the pathogenesis of ALK+ ALCL.[11,12,31,32] The oncogenic effect of NPM-ALK is dependent on STAT3,[30,37] STAT3 is constitutive activated in almost all ALK+ ALCL and

NPM-ALK transformed cells.[30,35,36] Constitutive activation of STAT3 is multifactorial; it involves JAK3, Src, and NPM-ALK.[33,34,50]

It is reported that loss of SHP1 expression due to gene hypermethylation has been identified to be common in hematopoietic malignancies.[74,96] Moreover, Khoury *et al*[52] investigated SHP1 expression in ALCL and found that SHP1 was silent due to gene hypermethylation in more than 80% of ALK+ ALCL cell lines and clinical tumor samples.

Wu *et al* restored SHP1 expression in a myeloid leukemia cell line by gene transfection and led to cell cycle arrest but no biochemical analysis. Two other study groups restored SHP1 expression by using the DNA methyltransferase inhibitor and this restoration of SHP1 was associated with a reduction in STAT3 activity.[78,97] Nevertheless, the role SHP1 played in the dysregulated STAT3 tyrosine kinase activity and oncogenesis remain to be elucidated. In view of the normal function of SHP1, we hypothesize that SHP1 can interact with NPM-ALK, and negatively regulate its tyrosine kinase activity and oncogenic potential. However, none of the published literature has addressed this issue.

We tested our hypothesis in two ways. First, we restored SHP1 expression by 5-AZA (5-aza-2'-deoxycytidine), an inhibitor of DNA Methyl-transferase and SHP1

gene transfection in SHP1-/ALK+ ALCL. Second, we blocked SHP1 expression by siRNA in SHP1+/ALK+ ALCL. Biological and biochemical changes associated with the STAT3 signaling in ALK+ ALCL cell lines were examined. Our main objective is to evaluate the biologic effects of the loss of SHP1 exerted on STAT3 signaling and oncogenesis in ALK+ ALCL.

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## Chapter 2

# Restoration of SHP1 expression by 5-AZA-2'-deoxycytidine is associated with downregulation of JAK3/STAT3 signaling in ALK-positive anaplastic large cell lymphoma

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✧ All the experiments presented in this chapter were done by Yajun Han except for the plasmid construction and the assay for the NPM-ALK tyrosine kinase activity

## Introduction

Anaplastic large cell lymphoma (ALCL) is a distinct type of aggressive non-Hodgkin's lymphoma characterized by CD30 expression, a sinusoidal infiltrative pattern and a T/null-cell immunophenotype.[1] Approximately 60% of these tumors have the chromosomal translocation, t(2;5)(p23;q35), that juxtaposes the nucleophosmin (NPM) gene at 5q35 with the anaplastic lymphoma kinase (ALK) gene at 2p23, leading to the expression of the aberrant fusion gene protein, NPM-ALK.[2] NPM-ALK is oncogenic; previous data suggest that it directly contributes to lymphomagenesis through deregulating multiple signaling pathways by virtue of its tyrosine kinase activity.[3-7] More recent studies have also implicated the signal transducer and activator of transcription 3 (STAT3) signaling pathway in the pathogenesis of ALK+ ALCL.[8-10] STAT3 itself is oncogenic,[11] and many types of human cancer demonstrate constitutive activation of STAT3.[12-15] It is believed that STAT3 promotes oncogenesis by upregulating a number of proteins that promote antiapoptotic effects or cell-cycle progression, such as Bcl-XL,Mcl-1, survivin and cyclin D3. It has been previously shown that ALK+ ALCL tumors consistently show relatively high levels of STAT3 activation,[8,9,16] and we have previously reported that specific blockade of STAT3 activation in ALK+ ALCL cell lines using a dominant-negative construct effectively triggers apoptosis and cell-cycle arrest, which correlates with downregulation of these STAT3 downstream targets.[10] More recently, Chiarile *et al* have provided further evidence to support the role of STAT3 in NPM-ALK-mediated tumorigenesis.

Although it is clear that NPM-ALK activates STAT3 in ALK+ ALCL cells, accumulating evidence suggests that other biochemical abnormalities also contribute to the high level of STAT3 activation seen in this cell type. Tyrosine kinases other than NPM-ALK have been shown to activate STAT3, such as src[18] and JAK3[9,19] with the latter being one of the normal physiologic activators of STAT3. Another group of defects is related to the loss of the negative regulators for the JAK/STAT signaling pathway such as SHP1.[20] SHP1, a non-transmembrane protein tyrosine phosphatase, is expressed primarily in hematopoietic cells.[21-23] It is known to function as an important negative regulator in various signal-transduction pathways including those of cytokine receptors (e.g. Epo-R, IL-3R, IL- 2R), growth factors with an intrinsic tyrosine kinase activity (e.g.CSF-1, EGF, c-Kit) and the antigen receptors of B and T cells.[24,25] SHP1 has been shown to silence the JAK/STAT pathway by dephosphorylating and inactivating JAK.[26,27] SHP1 has been reported to have tumor suppressor function.[30] In a previous study, we demonstrated that loss of SHP1 expression related to gene methylation is found in ALK+ ALCL cell lines and is detectable in the majority of ALK+ ALCL tumors.[31] Nevertheless, the role of SHP1 in ALK+ ALCL cells has not been extensively studied. The possible relationship between loss of SHP1 and the high levels of JAK3/STAT3 activation in this tumor type has been largely speculative.

We hypothesized that loss of SHP1 contributes to the constitutive activation of JAK3 and STAT3 in ALK+ ALCL cells. To test this hypothesis, we treated two ALK+ ALCL cell lines with 5-aza-2'-deoxycytidine (5-AZA), a DNA methyltransferase inhibitor, and correlated changes in the JAK3/STAT3 signaling pathway.

## Materials and methods

### Cell lines and tissue culture

ALK-positive ALCL cell lines, Karpas 299 and SU-DHL1, have been previously characterized and described.[32,33] These two cell lines were maintained in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), penicillin (10 000 U/ml; Sigma, St Louis, MO, USA), streptomycin (10 mg/ml; Sigma), and L-glutamine (200mM, Life Technologies). U266, a myeloma cell line, was also maintained in RPMI similar to that described for ALCL cell lines. GP293, an embryonic kidney cell line, was maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS and antibiotics (10 mg/ml streptomycin and 10 000 U/ml penicillin). All cell cultures were maintained under an atmosphere of 95% $O_2$ and 5% $CO_2$ in 98% humidity at $37^0$C.

### Pharmacologic agents

5-AZA was purchased from Sigma-Aldrich (Toronto, Ontario, Canada). 5-AZA was initially dissolved in acetic acid and diluted to 10 mg/ml. The aliquoted 5-AZA was frozen at $-20^0$C until being used. The final concentrations of 5-AZA used throughout this study were 0, 5 and 10 μM, and the use of these concentrations was based on the findings of two previous studies[34,35] as well as those of our preliminary studies. In all experiments, cells were seeded in six-well culture plates at a density of $10^6$/ml, and 5-AZA in fresh medium was added to the cell culture daily in order to maintain the constant concentration. Cells were harvested daily for 5 days for analysis.

MG132, purchased from Calbiochem (EMD Biosciences, San Diego, CA,USA), was initially dissolved in dimethyl sulfoxide (DMSO) and further diluted with sterile, de-ionized water to a final concentration of 1mM. The aliquoted MG132 was then stored at -20$^0$C. At the time of the experiment, MG132 was thawed and diluted with tissue culture media to a final concentration of 10 μM. AG490 was purchased from Calbiochem (San Diego,CA, USA) and diluted with DMSO (final concentration, 1mM).

**Western blot analysis and antibodies**

Western blot analysis was performed using standard techniques and details have been described previously.[19] All antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) unless otherwise specified: Bcl-2 (Dako, Carpinteria, CA, USA), Bcl-xL (Zymed, San Francisco,CA, USA), survivin (Novus Biologicals, Littleton, CO, USA), and actin (Sigma, St. Louis, MO, USA). All primary antibodies were used in a 1:1000 dilution.

**Cell cycle analysis using propidium iodide and flow cytometry**

Karpas 299 and SU-DHL-1 cells treated with 5-AZA were incubated with propidium iodide (Molecular Probes, Eugene,OR, USA) following the manufacturer's protocol. Briefly, the cells were washed and fixed in cold ethanol for 15 min and incubated with propodium iodide for 30 min. Thereafter, cells were analyzed with a flow cytometer (Becton Dickinson, San Jose, CA, USA).

**Reverse transcription-polymerase chain reaction**

Total cellular RNA was extracted by the RNeasy MINI Handbook Kit (Qiagen,

Valencia, CA, USA). Reverse transcriptase-PCR (RT-PCR) was performed using the one-step kit purchased from Qiagen following the manufacturer's protocol. Briefly, the reaction mixture, which was 50 μl in the final volume, consisted of 2μg RNA template and 0.6μM of forward and reverse primers for SHP1. The RT reaction consisted of one cycle of incubation for 30 min at $50^0$C, and the PCR consisted of 40 cycles of the following conditions: $95^0$C for 1 min, then $60^0$C for 1 min, followed by $72^0$C for 2 min. The final extension time was 10 min at $72^0$C. The SHP1 PCR primer was described previously: forward primer, 5'-CTCTCCGGAAGCCCCCAGGATG-3', and reverse primer, 3'-CCACCTGAGGACAGCACCGCT-3'.[36] The expected product size of the SHP1 amplicon is of 1749 bp. The sequences of the β-globin primers are as follows: (sense): 5'-CCTGGCTCACCTGGACAACCTCAA-3' and (antisense): 5'-TAGCCACACCAGCCACCACTTTCT-3'. The expected product size of the β-globin amplicon is of 190 bp. The amplified PCR products were then separated on 1% agarose gel and visualized with ethidium bromide stain.

**Measurement of ALK tyrosine kinase activity**

The activity of NPM-ALK tyrosine kinase was measured using a commercially available kit (Sigma, St Louis, MO, USA) and details of this method have been described previously.[19]

**Gene transfection of NPM-ALK**

The NPM-ALK cDNA was initially amplified using an NPM-ALK plasmid (a kind gift by Dr S Morris, St Jude' Children Research Hospital, Memphis, TN, USA) using a

primer set: ALK (forward) 5'-ATGGAAGATTCGATGGACATG-3' and NPM (reverse) 5'-TCAGGGCCCAGGCTGGTT-3'. The NPM-ALK cDNA was blunt ligated with pCruz His™, which was initially digested with EcoRV. The resulted vector was transformed into *Escherichia coli* on ampicillin-resistant plates, and plasmids were isolated by the Mini-prep kit (Qiagen). The sequence and orientation of the insert were confirmed by DNA sequencing. Transfection experiments were carried out in six-well plates using LipofectAMINE (Invitrogen Life Technologies) according to the manufacturer's protocol.

### Statistical analysis

Statistical analysis was performed by Student's t-test. A P-value of $<0.05$ was considered to be statistically significant.

## Results

### 5-AZA induced SHP1 expression in ALK+ ALCL cells

SHP1 was not detectable in either Karpas 299 or SU-DHL-1 at the steady state, as shown by Western blots and RT-PCR. As shown in Figure 2-1, SHP1 mRNA became detectable in Karpas 299 with addition of 5-AZA to the cell culture 24 h after the initiation of the experiment, with 10 μM of 5-AZA inducing a higher level than 5 μM. Western blot analysis showed that SHP1 protein was expressed in Karpas 299, first detectable 2 days after the initiation of the experiment (Figure 2-2a). The protein level of SHP1 increased gradually with time, reaching the highest level on day 5. On day 2, 10 μM of 5-AZA induced a higher level of SHP1 than 5μM, but there were no appreciable differences in the SHP1 level between these two

different dosages of 5-AZA from day 3 to day 5. Induction of SHP1 was similarly observed for SU-DHL-1, except that a higher level of SHP1 was induced with 10 μM compared to 5 μM, as illustrated in Figure 2-2b (day 5 results).

**SHP1 expression correlated with decreased expression of JAK3, pJAK3 and pSTAT3**

We then evaluated if the restoration of SHP1 expression is associated with any changes in the JAK3 tyrosine phosphorylation in both ALK+ ALCL cell lines. As illustrated in Figure 2-2a and c, no substantial changes in the expression of JAK3 and pJAK3 were identified shortly after treating Karpas 299 cells with 5-AZA. Nevertheless, both JAK3 and pJAK3 markedly decreased on day 3 when compared to cells treated with diluent only (negative controls). When the pJAK3 levels were assessed after correction of the total JAK3 was made, we detected that pJAK3 was downregulated more than that of the total JAK3 (Figure 2-2c).The decreased levels of JAK3 and pJAK3 inversely correlated with the marked increase in the SHP1 protein level on day 3. Whereas there were no appreciable changes in the total STAT3 protein level throughout the experiment, pSTAT3 decreased with 5-AZA. Compared to the decreases in JAK3 and pJAK3, the decrease in pSTAT3 was delayed, with >50% reduction identifiable on day 5 of treatment with 10 μM of 5-AZA (Figure 2-2a and c). Similar results were obtained from SU-DHL-1 cells treated with 5-AZA (illustrated in Figure 2-2b). To ensure these changes were not owing to a nonspecific drug effect of 5-AZA, we performed similar experiments using U266 cells, a myeloma cell line that we have confirmed to have constitutive activation of STAT3 and SHP1 expression at the steady state. No detectable

changes in SHP1, pSTAT3 and STAT3 were identified in U266 cells treated with 5-AZA for 5 days. These findings support a link between the restoration of SHP1 expression and changes in the JAK3/STAT3 pathway.

**Decreased STAT3 activation was associated with modulation of some but not all STAT3 downstream targets**

We have previously shown that specific blockade of STAT3 in ALK+ ALCL cells using a dominant-negative construct induced significant alterations in known downstream targets of STAT3 signaling, including Bcl-2, Mcl-1, SOCS3, survivin and cyclinD3.[10] Thus, we assessed if the decrease in pSTAT3 associated with SHP1 expression is sufficient to induce similar changes in these STAT3 downstream targets. As illustrated in Figure 2-2d, the expression levels of cyclin D3 and survivin showed no detectable changes throughout the experiment, but Mcl-1,SOCS-3 and Bcl-2 showed concentration-dependent and time-dependent decreases in their protein levels after treatment with 5-AZA. The decrease in the protein level of Bcl-2 was first noted on day 3, whereas the decreases in SOCS-3 and Mcl-1 were first detectable on day 5. Changes in the STAT3 downstream targets for SU-DHL-1 cells are also illustrated in Figure 2-2d (right panel). The overall pattern was similar although more marked downregulation in cyclin D3 and Mcl-1 was noted. Similar experiments were performed in U266; no detectable changes were noted in these targets.

**Proteasome inhibitor, MG132, inhibited 5-AZA-induced downregulation of JAK3, pJAK3 and STAT3**

SHP1 has been reported to inhibit JAKs not only by tyrosine dephosphorylation, but

also by decreasing the total protein level via the proteasome pathway.[37] Thus, we tested if proteasome inhibitor, MG132, can inhibit the 5-AZA-induced downregulation of the total JAK3 protein level. Both Karpas 299 and SUDHL-1 cells were incubated in the presence of 5 μM of 5-AZA for 4 days, treated with MG132 (10 μM) and harvested at 0, 2, 6 and 10 h after the addition of MG132. As illustrated in Figure 2-3, JAK3, which was present at a low level after 4 days of 5-AZA treatments, increased in expression in a time-dependent manner. SHP1, pJAK3 and pSTAT3 all increased in their levels with the addition of MG132.

**5-AZA did not induce significant changes to the ALK tyrosine kinase activity**

As we previously showed that JAK3 might potentiate the tyrosine kinase activity of NPM-ALK in ALK+ ALCL cell lines,[19] we tested whether there are changes in the tyrosine kinase of NPM/ALK on day 4, during which both JAK3 and pJAK3 had been downregulated. As shown in Figure 2-4, in vitro tyrosine kinase assay showed that Karpas 299 cells treated with 5 μM of 5-AZA for 4 days induced a slight increase in ALK enzymatic activity to 120% of its baseline level, although this difference is not statistically significant ($P>0.05$). In contrast, a selective JAK inhibitor, AG490, significantly reduced the ALK enzymatic activity to approximately 42% of the negative control cells (acetic acid only).

**5-AZA induced cell-cycle arrest and sensitized ALCL cells to doxorubicin-induced apoptosis**

Compared to untreated Karpas 299 and SU-DHL-1 cells, treatment with 5-AZA induced a significant decrease in the cell viability as assessed by Trypan blue staining. As shown in Figure 2-5a, 5-AZA induced a significant decrease in the

number of viable cells in both cell lines compared to the untreated cells. As illustrated in Figure 2-5b, cell cycle analysis revealed that there was a decrease in the proportion of Karpas 299 cells in the S phase as well as the G2/M phase. The S-phase fraction decreased from 31 (untreated) to 22 and 21%, at 5 and 10 μM of 5-AZA, respectively. There was no detectable increase in the sub-G0/G1 fraction. The lack of an increase in apoptosis was further supported by the absence of a detectable increase in the cleaved caspase-3 level shown on Western blots (not shown). Despite the absence of increased apoptosis, 5-AZA potentiated apoptosis induced by doxorubicin, a chemotherapeutic drug used commonly to treat ALCL patients. As shown in Figure 2-5c, Karpas 299 cells were treated with a relatively low dose (0.1 μM) of doxorubicin. Compared with the use of doxorubicin alone, the combination of doxorubicin and 5-AZA was more effective in reducing the number of viable cells, although the difference between the two experimental conditions is statistically significant only on day 4.

In view of a recent publication that active STAT3 binds to DNA methyltransferase 1 (DNMT1) and promotes methylation/silencing of SHP1,[38] we tested if transfection of NPM-ALK, which activates STAT3 in ALK+ ALCL cells, leads to downregulation of SHP1. After gene transfection into GP293 cells, STAT3 became tyrosine phosphorylated and SHP1 was downregulated (Figure 2-6), and the decrease was estimated to be 32% using densitometry analysis (after correction of a slight difference between the actin bands). Interestingly, the total STAT3 band was consistently decreased slightly (averaging 88% of the negative control).

## Discussion

Loss of SHP1 associated with gene hypermethylation is a relatively common abnormality in hematologic cancers;[31,40,41] the exact biological significance of this abnormality has not been extensively studied. The main objective of this study is to correlate the restoration of SHP1 expression using 5-AZA and changes in the JAK3/STAT3 signaling pathway in ALK+ ALCL cells, which are often characterized by the absence of SHP1 and constitutive activation of the JAK3/STAT3 pathway.

We found that 5-AZA induced a time-dependent increase in the SHP1 mRNA and protein level in both ALK+ ALCL cell lines, and this change coincided with the downregulation of pJAK3 and JAK3, followed by a decrease in pSTAT3. Substantial (i.e. >50% of the negative controls) downregulation of both JAK3 and pJAK3 was detectable. The finding that pJAK3 did not decrease in their levels until day 3 of the experiment is rather unexpected, as the current concept is that SHP1 is a rapid acting tyrosine phosphatase. This unexpected result suggests that the initially low level of SHP1 is not sufficient in balancing the overactivated JAK3. We also found that reduction in the total JAK3 protein level was dependent on the proteasome degradation pathway, as a proteasome inhibitor, MG132, efficiently blocked the decrease of JAK3. A parallel observation was made by Wu *et al* [37], who found that expression of SHP1 in HTB26 cells negatively regulates JAK1 by dephosphorylation and promotion of proteasome degradation. Thus, SHP1 negatively regulates the JAK/STAT pathway through at least two different mechanisms. Additional studies are required to delineate how SHP1 promotes proteasome degradation of JAK3. Interestingly, as shown in Figure 2-3, both

pSTAT3 and SHP1 increased in their levels after addition of MG132, and these findings support the concept that STAT3 activation is more related to the expression/activation level of JAK3 than the SHP1 expression. Another interesting observation is that a marked reduction of the pSTAT3 level was relatively delayed compared to that of JAK3 or pJAK3. This finding suggests that SHP1 downregulated STAT3 activation indirectly, likely via decreasing JAK3 expression and activation. This is in keeping with our previous finding that JAK3 contributes to STAT3 activation in ALK+ ALCL cells.[18]

Zhang *et al* [38] recently reported that active STAT3 forms a complex with DNMT1, which binds to the SHP1 promoter and facilitates gene methylation of SHP1. As NPM-ALK is an important activator of STAT3 in ALK+ ALCL, we tested if ectopic NPM-ALK expression in GP293 cells results in downregulation of SHP1. Results from our gene transfection experiments (Figure 2-6) are entirely in keeping with this concept. However, our findings suggest that STAT3 activation alone is not sufficient to induce complete SHP1 silencing; additional mechanisms likely exist to achieve complete SHP1 gene silencing. A mild decrease in the total STAT3 level was identified after NPM-ALK; the mechanism underlying this finding is unclear.

The partial but incomplete downregulation of pSTAT3 after 5-AZA treatment is expected, as the other activators of STAT3 such as NPM-ALK and src are probably functional. With the partial decrease in the pSTAT3 level, a number of STAT3 downstream targets showed detectable decreases in their expression (such as Mcl-1, SOCS3 and Bcl-2). The relative lack of changes in survivin and cyclin D3 in

this study is probably owing to the persistence of STAT3 activation, albeit at a relatively low level. These findings suggest that the expression of various STAT3 downstream targets may be triggered at different levels of STAT3 activation.

Despite the decrease in Bcl-2 and Mcl-1 levels, we did not detect significant apoptotic cell death with 5-AZA. Lack of apoptotic cell death in our experiments can at least be partially explained by the persistent expression of survivin. Survivin is an important anti-apoptotic protein.[41] Previous studies have shown that STAT3 induces its anti-apoptoic effects via induction of survivin,[42] and enforced expression of survivin can suppress cell death induced by STAT3 inhibition.[43] Nevertheless, we found that 5-AZA sensitized Karpas 299 cells to doxorubicin-induced apoptosis, and this may be due to decreases in other antiapoptotic proteins such as Mcl-1 and Bcl-2. In support of this concept, the difference in apoptosis between Karpas 299 cells treated with combined 5-AZA and low-dose doxorubicin and cells treated with low-dose doxorubicin alone was not statistically significant until day 4 of the experiment, and this correlates with the findings that Bcl-2 and Mcl-1 did not show substantial decreases in their expression level until day 4 or 5,respectively.

We found that 5-AZA induced cell-cycle arrest in Karpas 299 cells, predominantly in the G2/M phase. This is almost certainly not related to cyclin D3, a G1 promoter that did not show significant changes in its expression level after 5-AZA. From the literature, it has been reported that the p38/MAPK pathway is modulated by 5-AZA,[44] and downregulation of this pathway may be responsible for the G2/M arrest observed in this study. As 5-AZA can modulate the expression of many genes, depending on the gene methylation pattern of specific cell types, it is highly

likely that other genes that may have been upregulated by this agent in ALK+ ALCL cells are responsible for these biological effects.

Lastly, after we submitted this manuscript for publication, Honorat *et al*[45] have published that NPM-ALK is a substrate for SHP1, and SHP1 negatively regulates NPM-ALK and decreases tumorigenecity. Thus, these findings are in keeping with the concept that loss of SHP1 contributes to the pathogenesis of ALK+ ALCL. As mentioned in discussion of this paper, there is a discrepancy related to the SHP1 expression in Karpas 299 cells between the two studies. We believe that this discrepancy is likely owing to the existence of different subclones of this cell line.

## Conclusions

We found that restoration of SHP1 expression induced by 5-AZA correlates with a significant downregulation of the JAK3/STAT3 signaling in both ALK+ ALCL cells. Our data support the concept that loss of SHP1 contributes to the pathogenesis of ALK+ ALCL. In addition to its role as a tyrosine phosphatase, SHP1 silences JAK3 by virtue of promoting JAK3 degradation via the proteasome pathway.

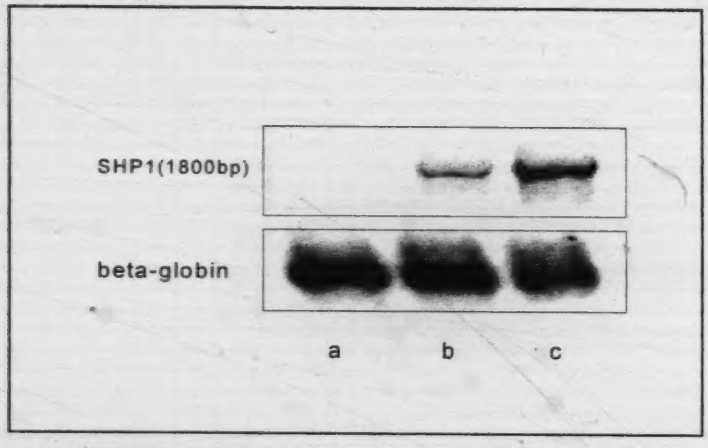


**Figure 2-1** RT-PCR assay to measure the SHP1 mRNA. Whereas no SHP1 was detectable in Karpas 299 cells at the steady state (lane a), 5-AZA treatment induced expression of SHP1 24 h after initiation of the experiment, and 5 μM of 5 AZA (lane b) induced a lower level than 10 μM did (lane c).

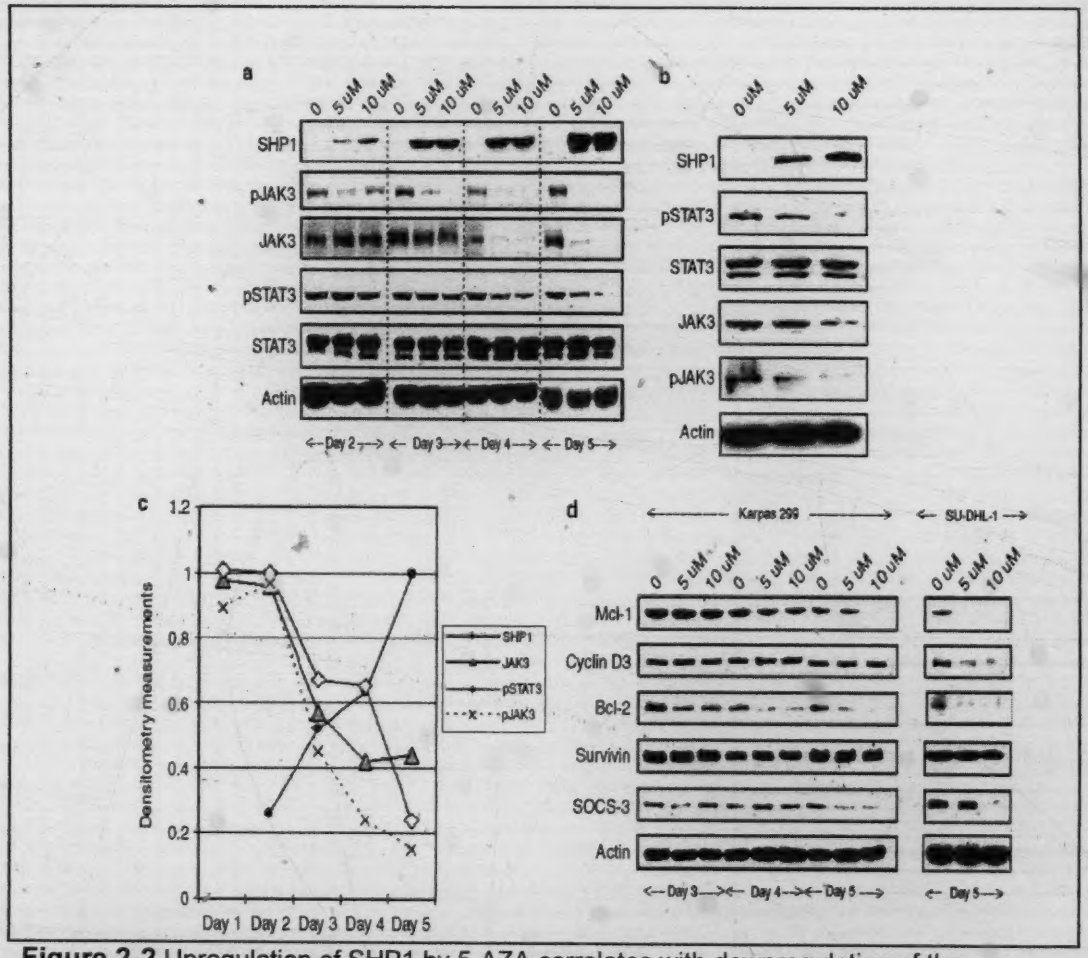


**Figure 2-2** Upregulation of SHP1 by 5-AZA correlates with downregulation of the JAK3/STAT3 signaling in ALK+ ALCL cells. (a) Western blot analysis showed that 5-AZA induced time-dependent increase in SHP1 in Karpas 299 cells. In the same experiment, decreases in JAK3, pJAK3 and STAT3 were identified, although the decrease in pSTAT3 was relatively delayed. (b) Western blot analysis revealed that SU-DHL-1 cells treated with 5-AZA in 5 days showed restoration of SHP1 and downregulation of pJAK3, JAK3 and pSTAT3, similar to the changes seen for Karpas 299 cells. (c) Densitometric measurement of the band intensity for pJAK3, JAK3 and pSTAT3 shown in (a). For each day, the band intensity of SHP1, JAK3, pSTAT3 and pJAK3 in cells treated with 10 μM of 5-AZA was normalized to that from cells treated with solvent only (negative controls). pJAK3 was also normalized to the level of JAK3 for each drug dosage and each day. Compared to the untreated samples, pJAK3 decreased more than JAK3, and substantial decrease (i.e. >50% reduction) of pSTAT3 was identified later than that of JAK3 and pJAK3. (d) Western blot analysis showed that 5-AZA induced decreases of some but not all of the STAT3 downstream targets in Karpas 299 cells. Mcl-1, Bcl-2 and SOCS-3 were downregulated, whereas cyclin D3 and surviyin showed no detectable changes. A similar pattern of changes was identified in SU-DHL-1 cells, except that downregulation in cyclin D3 was also detected in this cell line.

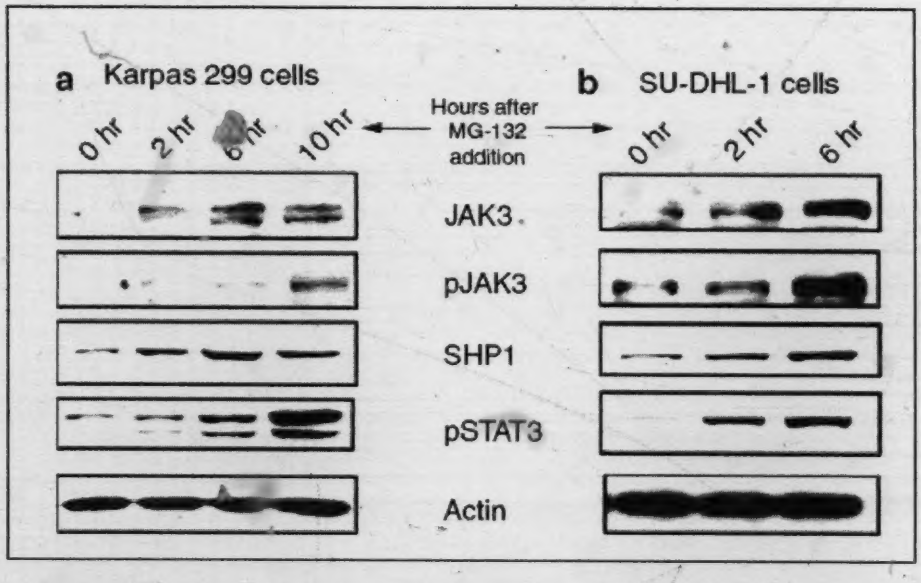


**Figure 2-3** MG132 blocked downregulation of JAK3 induced by 5-AZA. Karpas 299 cells and SU-DHL-1 cells incubated with 5-AZA for 4 days were treated with MG132, a proteasome inhibitor. MG132 induced time-dependent upregulation of JAK3, pJAK3 as well as pSTAT3, in spite of the concomitant increase of SHP1.

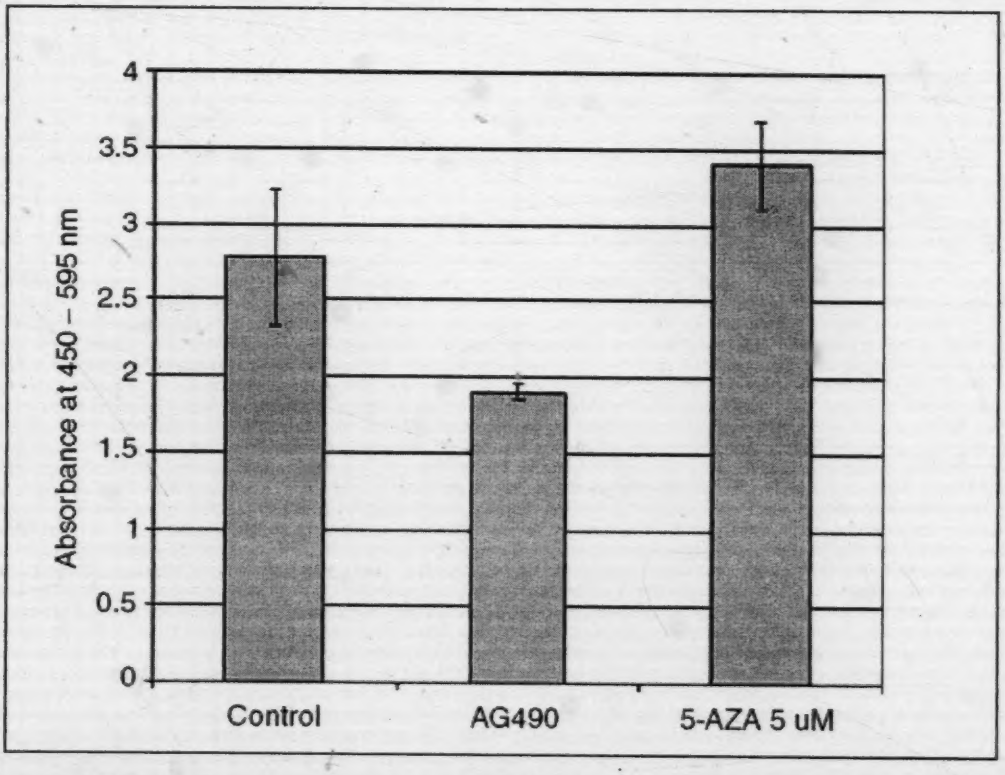


**Figure 2-4** In vitro measurement of the NPM-ALK tyrosine kinase activity after 5-AZA treatment. 5-AZA did not significantly decrease the absorbance in Karpas 299 compared to the negative controls (treated with acetic acid only). AG490, which was previously shown to decrease the tyrosine kinase activity of NPM-ALK, served as a control.

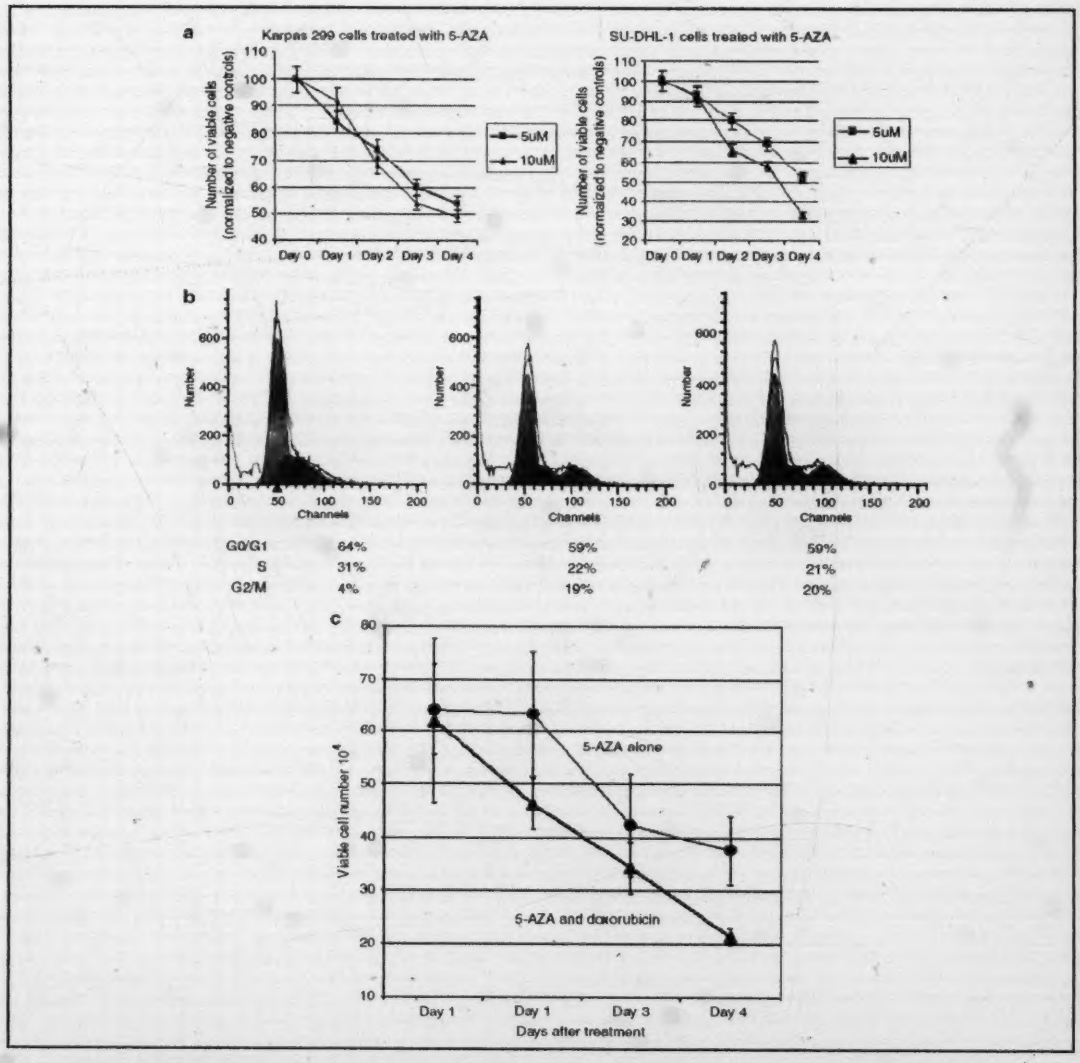


**Figure 2-5** Biological effects of 5-AZA in ALK+ ALCL cells. (a) Trypan blue exclusion test in Karpas 299 cells treated with 5-AZA: Karpas 299 cells were treated with solvent only (negative controls), 5 μM of 5-AZA or 10 μM of 5-AZA. The cell numbers of 5-AZA-treated cells were normalized to that of the negative controls. 5-AZA significantly decreased the number of viable cells. No significant difference was seen between the two dosages of 5-AZA. Triplicate experiments were performed. (b) Cell cycle analysis was performed using Karpas 299 cells treated with diluent (left) or 5-AZA (5 μM, middle; 10 μM, right) for 4 days. Cells were analyzed by flow cytometry after staining with propidium iodide. Compared to the negative controls, treated cells showed increased proportion of cells in the G2/M phase. (c) 5-AZA potentiated the apoptosis induced by doxorubicin: karpas 299 cells treated with a combination of low-dose doxorubicin (0.1 μM) and 5-AZA induced more cell death than low-dose doxorubicin alone. The number of viable cells was assessed by Trypan blue exclusion assay. Triplicate experiments were performed.

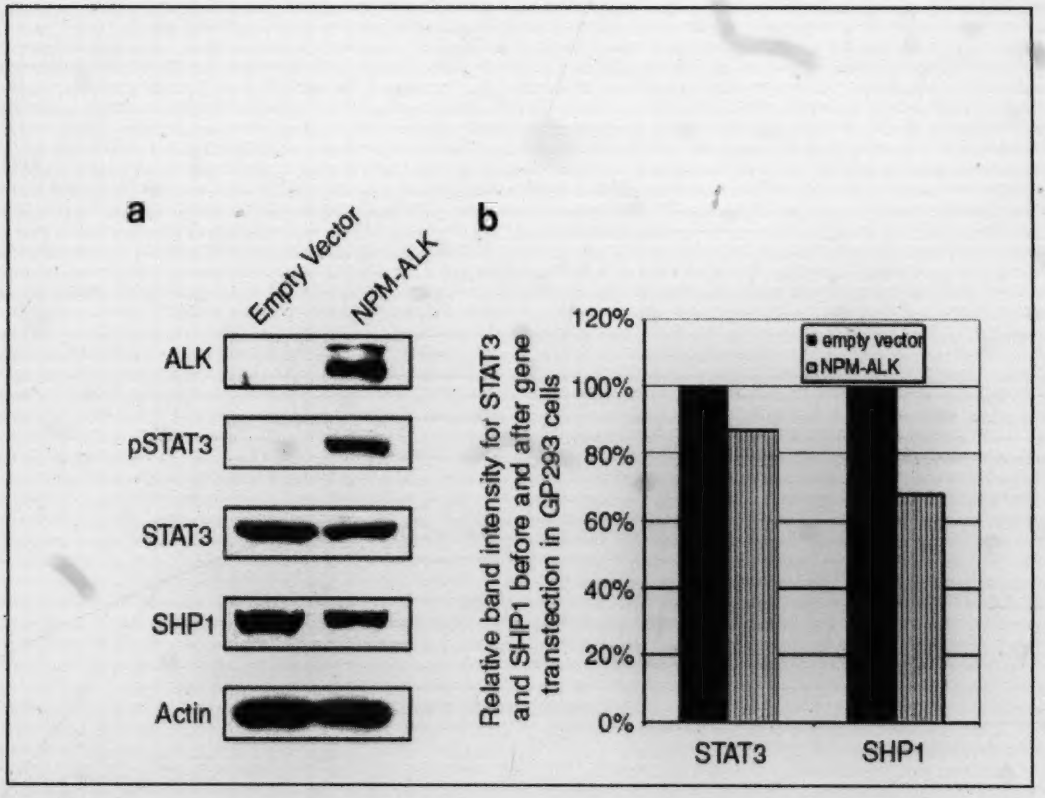


**Figure 2-6** Transfection of NPM-ALK induced a partial downregulation of SHP1. GP293 cells were transfected with an empty vector or an NPM-ALK-expressing vector. Cells transfected with NPM-ALK showed evidence of STAT3 activation (i.e. expression of pSTAT3) and a partial decrease in SHP1 expression, which was shown to be a 32% reduction by densitometry analysis. A relatively slight decrease in the total STAT3 band was also detectable after NPM-ALK transfection.

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# Chapter 3

# Loss of SHP1 Enhances JAK3/STAT3 Signaling and Decreases Proteosome Degradation of JAK3 and NPM-ALK in ALK-positive Anaplastic Large Cell Lymphoma

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- ✧ All the experiments presented in this chapter were done by Yajun han except the plasmid construction, immunohistochemistry, and quantitative PCR assay for SHP1.

## INTRODUCTION

SHP1 is a non-transmembrane protein tyrosine phosphatase expressed most abundantly in hematopoietic cells.[1] It serves as an important negative regulator in cytokine-mediated signal transduction, including that of the JAK/STAT signaling pathway.[2-7] Gene methylation and silencing of SHP1 are relatively common in hematologic neoplasms, and a significant proportion of lymphoid malignancies of both B- and T-cell lineages are negative for SHP1 protein expression.[8-10] In view of its normal function, loss of SHP1 has been hypothesized to deregulate various signaling pathways and promote lymphomagenesis. Nevertheless, relatively few studies have been performed to directly test this hypothesis and delineate the mechanism by which loss of SHP1 contributes to lymphoma formation. One previous study has shown that transfection of SHP1 in a myeloid leukemia cell line leads to cell cycle arrest, but the biochemical basis of these SHP1-induced changes was not fully examined.[12] Using a DNA methyltransferase inhibitor, Chim *et al* [13] reported that SHP1 expression in myeloma cell lines correlated with a reduction in the tyrosine phosphorylation of STAT3. However, DNA methyltransferase inhibitors are not gene-specific,[14] and the relationship between SHP1 expression and downregulation of STAT3 activation cannot be definitely established. In addition to its function as a tyrosine phosphatase, SHP1 also has been implicated in the regulation of the rate of proteosome degradation of certain cellular proteins,[15,16] but the biological significance of this relatively novel function

of SHP1 has not been extensively studied in cancers.

ALK-positive anaplastic large cell lymphoma (ALK+ALCL) is a distinct type of non-Hodgkin's lymphoma, characterized by CD30 expression, a sinusoidal infiltrative pattern, T/null cell immunophenotype, and the expression of the oncogenic fusion chimeric protein, NPM-ALK.[17,18] NPM-ALK is the result of the chromosomal translocation, *t*(2;5)(p23;q35), that juxtaposes the nucleophosmin (NPM) gene at 5q35 with the anaplastic lymphoma kinase (ALK) gene at 2p23.[19] Recent studies have shown that NPM-ALK directly contributes to the pathogenesis of ALK+ALCL, by exerting its tyrosine kinase activity embodied in the ALK portion of the fusion protein and thereby deregulating multiple signaling pathways.[20-24] One of these signaling pathways is the STAT3 pathway.[25-27] STAT3 has been shown to be oncogenic in a wide variety of human cancers,[28-33] and there is ample evidence that STAT3 activation is crucial to the pathogenesis of ALK+ALCL.[25,27,34] While it is likely that NPM-ALK mediates oncogenesis via STAT3 activation, previous data also suggest that activation of STAT3 in ALK+ALCL is multi-factorial. For instance, we previously showed that JAK3 (a physiologic activator of STAT3) is frequently activated in ALK+ ALCL,[35] and JAK3 contributes to STAT3 activation in ALK+ALCL.[36] Zhang *et al* [26] provided evidence to support the model of 'multi-level dysregulation of STAT3 activation' in ALK+ALCL. With regard to SHP1, one of our previous studies have identified

gene methylation of SHP1 and loss of SHP1 expression in the vast majority of ALK+ALCL tumors.[37] Since SHP1 has been implicated in the JAK/STAT signaling, it is possible that loss of SHP1 potentiates JAK3/STAT3 signaling in ALK+ALCL cells, and contributes to the lymphomagenesis in this cell type. This hypothesis, however, has not been directly tested. The less understood role of SHP1 as a modulator of proteosome degradation also has not been examined in ALK+ALCL.

To directly assess the biological importance of loss of SHP1 in ALK+ALCL, we transfected two ALK+ALCL cell lines, Karpas 299 and SU-DHL-1, with specific SHP1 expression vectors. In view of the central pathogenetic roles of NPM-ALK, JAK3 and STAT3 in these tumors, we focused our analysis on these proteins with regard to their expression and/or tyrosine phosphorylation status. Whether restoration of SHP1 expression in ALK+ALCL cells can modulate degradation of these proteins was evaluated. These changes were then correlated with changes in cell proliferation and apoptosis.[6]

## METHODS AND MATERIALS

### Cell lines, tissue culture, and reagents

Three ALK+ALCL cell lines, Karpas 299, SU-DHL-1, and SUP-M2, were included in this study. These cell lines were maintained at 37°C in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated fetal

bovine serum, 10,000 units/mL penicillin (Sigma, St. Louis, MO), 10 mg/mL streptomycin (Sigma) and 200 mM of L-Glutamine (Life Technologies) in a humidified atmosphere containing 5% CO2 and 95% O2. MG132, purchased from Calbiochem (EMD Biosciences, Inc. San Diego, CA, USA), was initially dissolved in DMSO and further diluted with sterile, de-ionized water to a final concentration of 1 mM. The aliquoted MG132 was then stored at –20°C. During the experiment, MG132 was thawed and diluted with tissue culture media to a final concentration of 10 μM.

**Plasmid constructs and cell transfection**

Two different SHP1 plasmids were constructed and used in this study. Full length SHP1 cDNA was initially synthesized using a primer set including the 5' end of SHP1 (Xho1)-F: 5'-AAGCCTCGAGGATGGTGAGGT -3' and the 3' end of SHP1(EcoR1)-R: 5'-GCGGAATTCTTCCACAGGGTCA-3' (Qiagen, Valencia, CA). Subsequently, the full length SHP1 cDNA was cloned into pCI (Promega, Madison, WI, USA) and pIRES2-EGFP (Clontech, BD Bioscience, Mountain View,CA). After amplification using a kit from Qiagen, the sequence and orientation of the SHP1 insert were confirmed by DNA sequencing using the ABI 3100 gene sequencer (San Jose, CA). The pIRES2-EGFP plasmid contains an internal ribosomal entry site which allows translation of SHP1 and GFP independently from a single bicistronic mRNA. Cell transfection was performed

using a commercially available transfection instrument (Amaxa, Koeln, Germany), following the manufacturer's recommended protocol. Specifically, $2x10^6$ SU-DHL-1 cells were transfected with 5 µg of vector (SHP1 or empty) in 100 µL of NucleofectorTM V-solution (Amaxa). Transfection for Karpas 299 cells was done similarly, except that only 3 µg of vector was used to achieve the most optimal level of transfection.

**siRNA to block SHP1 expression**

siRNA specific for SHP1 was purchased from Qiagen (Qiagen). SUP-M2, an ALK+ ALCL cell line confirmed to be SHP1-positive by RT-PCR and Western Blot in our study, was transfected with siRNA using the Amaxa instrument following the same transfection protocol described above. Transfected cells were cultured for 30 hours, and cell lysates were prepared and subjected to Western blot analysis.

**Flow cytometry and cell sorting**

The pIRES2-EGFP and pIRES2-EGFP-SHP1 transfected, ALK+ALCL cells were subjective to cell cycle analysis as well as cell sorting. For cell cycle analysis, transfected cells were re-suspended in a phosphate buffered saline (PBS)-EDTA buffer, stained with Draq 5 (Biostatus Ltd. Shepshed Leicestershire, UK) according to the manufacturer's protocol, and analyzed using the FACSort

cytometer (Becton Dickinson, San Jose, CA, USA). The results were then analyzed using the Cell Quest Software (Verity Software House Inc., Topsham,ME). For cell sorting, SHP1-transfected SU-DHL-1 cells were washed twice, high speed sorted using the EPICS ALTRA (Beckman-Coulter, Mississauga, Ontario, Canada), and collected into fetal bovine serum. Purities of >98% were achieved on analysis of the sorted cell population. The cells were washed with PBS and centrifuged at 1500 rpm for 10 min. The supernatant was aspirated and the cell pellet was frozen at -80°C. All of the pIRES2-EGFP or pIRES2-EGFP-SHP1 transfected SU-DHL-1 cells utilized throughout this study were cell-sorted and purified.

**Western blot analysis**

Western blot analysis was performed using standard techniques. Briefly, the cells were washed in phosphate buffered saline (PBS, pH=7.5) and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% sodium dodecaylsulfate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotonin (5μg/mL), sodium vanadate (1 mM) and leupeptin (5 μg/mL). After incubation on ice for 30 minutes, the lysates were subjected to centrifugation at 12,000 rpm for 10 minutes at 4 $^{0}$C. The supernatants were collected after the centrifugation. Protein concentration was determined with a protein assay kit (Bio-Rad, Hercules,CA, USA). Each lane of a 6% or 12% polyacrylamide gel was loaded with 80 μg of

protein. After electrophoresis and transfer to nitrocellulose membranes (Bio-Rad) by electroblotting, blots were probed with specific primary and secondary antibodies and the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) according to the manufacturer's protocol. The antibodies used in this study were those reactive with SHP1, STAT3, phospho-STAT3 (pSTAT3), JAK3, phospho-JAK3 (pJAK3), ALK1, cyclin D3, Mcl-1, Bcl-2, and PARP, all of which were purchased from Santa Cruz Biotechnology (Santa Cruz,CA, USA) and used at a dilution of 1:500. Antibodies reactive with beta-actin (Sigma), survivin (Sigma), active caspase 3 (Abcam, Cambridge, MA, USA), and NPM1 (NeoMarkers, Fremont, CA) were also used at a dilution of 1:500.

**Quantitative PCR assay to detect SHP1 in ALK+ALCL cell lines**

Total cellular RNA was extracted using the RNeasy MINI Handbook Kit (Qiagen). We performed quantitative RT-PCR using a method adapted from a previous report.[38] One-step RT-PCR was performed using the TaqMan Gold RT-PCR kit according to the manufacturer's protocol. Total RNA (50 ng) from Karpas 299, SUP-M2 and SU-DHL-1 cells was used as template. The SHP1 primer and probe sequences were as follows: forward: GGAGTCGGAGTACGGGAACAT, reverse: ATCCTCCTTGTGTTTGGACGA, probe: CCCCAGCCATGAAGAATGCCCA. The probe was labeled with FAM reporter dye on 5' end and measured at 518 nm wavelength, and the reporter dye was quenched by TAMPRA on the 3' end. The

TaqMan GAPDH control reagents were used. The thermal cycler parameters for SHP1 included 48°C for 30 minutes, 95°C for 10 minutes, and 40 cycles of denaturation (95°C for 15 seconds) and annealing/extension (54°C for 1 minute). The thermal cycler parameters for GAPDH were identical to that for SHP1, except that the annealing/extension condition was 60°C for 1 minute.

### ALK+ALCL tumor samples and immunohistochemistry

A total of 17 cases of ALK+ALCL were retrieved randomly from the file at the Department of Laboratory Medicine and Pathology, Cross-Cancer Institute, Edmonton, Alberta, Canada. The use of these tissues was approved by our institutional ethics committee. All cases were routinely fixed in formalin and embedded in paraffin. Immunohistochemistry was performed and details of the method have been described elsewhere.[33] Antibodies employed included anti-Mcl-1 (sc-819), anti-pJAK3 (sc-16567) and anti-SHP1 (sc-7289), all of which were purchased from Santa Cruz.

## RESULTS

### SHP1 expression in ALK+ALCL cell lines

We first confirmed the status of SHP1 expression in three ALK+ALCL cell lines

including Karpas 299, SU-DHL-1 and SUP-M2. As shown in Figure 3-1, Karpas 299 and SU-DHL-1 cells showed no detectable SHP1 protein expression on Western blots, whereas SUP-M2 cells expressed SHP1 relatively strongly. Of note, SUP-M2, the SHP1-positive ALK+ALCL cell line, had a relatively lower expression of pSTAT3, pJAK3 and JAK3 compared to the two SHP1-negative ALK+ ALCL cell lines. No appreciable differences were identified in the protein levels of STAT3 and NPM-ALK among these three cell lines. We also performed quantitative RT-PCR assay to measure SHP1 mRNA and the results correlated with those of Western blots, with the average threshold cycle for SUP-M2 being 29, and those for Karpas 299 and SU-DHL-1 being >40 (undetectable range).

**SHP1 expression resulted in downregulation of the JAK3/STAT3 pathway**

To directly assess the biological roles of SHP1 in ALK+ALCL cells, SU-DHL-1 was transfected with pIRES2-EGFP or pIRES2-EGFP-SHP1. Based on the expression of GFP analyzed by flow cytometry, the transfection efficiency was between 40-65%, with a median of 50% for both vectors (based on five independent experiments). Negative controls, which were SU-DHL-1 cells transfected with a PCI empty vector, showed no evidence of GFP-expressing cells.

Based on the GFP expression, transfected SU-DHL-1 cells were sorted using

flow cytometry, as described in Methods and Materials, and the purity of GFP expressing cells was confirmed to be >98%. Western blots analysis was performed comparing sorted cells transfected with pIRES2-EGFP and those transfected with pIRES2-EGFP-SHP1. As shown in Figure 3-2a (left panel), SHP1 was highly expressed in cells transfected with pIRES2-EGFP-SHP1 but absent in those transfected with pIRES2-EGFP. Expression of SHP1 was associated with substantial decreases in the protein levels of pJAK3, JAK3, and pSTAT3. Triplicate experiments were performed and results from a representative experiment are illustrated.

Although Karpas 299 cells were also transducible with the pIRES2-EGFP-SHP1 plasmid, the transfection efficiency, based on the expression of GFP analyzed by flow cytometry, was only 10% of the cells. Nevertheless, we found that a relatively high transfection efficiency could be obtained if pCI-SHP1 was used instead of pIRES2-EGFP-SHP1. Western blot analysis was performed to compare Karpas 299 cells transfected with pCI-SHP1 and those transfected with the empty vector pCI. Triplicate experiments were performed and results from a representative experiment are illustrated in Figure 3-2a (right panel), the expression levels of pSTAT3, pJAK3 and JAK3 were decreased in pCI-SHP1 transfected cells compared to the empty pCI-transfected cells. The decrease in pJAK3 was more obvious than that of JAK3. The pattern of changes in the expression of JAK3,

pJAK3 and pSTAT3 was thus similar to that seen in SHP1 transfected SU-DHL-1, although the changes were not as prominent in Karpas 299 cells, which can be explained by the fact that cell sorting was used for SUDHL-1 cells but not for Karpas 299 cells. Compared to that of SU-DHL-1 cells, the short form of pSTAT3 was expressed at a relatively low level in Karpas 299 cells, and this band became undetectable after SHP1 transfection in these cells.

We then determined if these alternations of the JAK3/STAT3 pathway lead to any significant changes in the expression of STAT3 downstream targets, previously shown to be decreased when the STAT3 activity was inhibited by a STAT3 dominant negative construct.[27] These targets often are involved in either cell cycle progression or apoptosis, included survivin, cyclin D3, Mcl-1, Bcl-2 and survivin. As shown in Figure 3-2b, compared to the negative controls, SU-DHL-1 and Karpas 299 cells transfected with pIRES2-EGFP-SHP1 or pCI-SHP1, respectively, showed downregulation of Mcl-1, Bcl-2 and cyclin D3. Relatively small decreases in survivin were also detected in both cell lines. The pattern of changes was again similar between SU-DHL-1 and Karpas 299, with more prominent changes seen in SU-DHL-1 cells, again likely due to the fact that cell sorting was done with SU-DHL-1 but not Karpas 299 cells.

Although NPM-ALK is not known to be a STAT3 downstream target, we

examined if SHP1 expression modulates the expression of this oncogenic protein, which has a central pathogenetic role in ALK+ALCL. As shown in Figure 3-2b, NPM-ALK was substantially downregulated in both cell lines after SHP1 transfection, compared to their negative controls. To further confirm the role of SHP1 in down-regulating JAK3/STAT3 signaling and the expression of NPM-ALK, we blocked SHP1 expression in SUP-M2, an ALK+ALCL cell line that expresses a relatively high level of SHP1 at the steady state. As shown in Figure 3-2c, siRNA decreased SHP1 expression, which was associated with upregulation of pSTAT3, JAK3 and pJAK3. NPM-ALK and the downstream effectors of STAT3 (such as cyclin D3) were also increased as the SHP1 protein expression was decreased.

**3) SHP1 decreased expression of NPM-ALK and JAK3 via the proteosome pathway**

We then questioned if the SHP1-mediated down-regulation of NPM-ALK and JAK3 is related to the proteosome pathway. SU-DHL-1 cells were treated with 10μM of MG132 at 24 hours after gene transfection with pIRES2-EGFP-SHP1 and cell sorting. As shown in Figure 3-3a, the initial downregulation of both JAK3 and NPM-ALK was reversed at 3-5 hours after the addition of MG132. However, MG132 did not induce any changes in the pJAK3 level in the same experiment. To support that the effects of MG132 in our experiments are not due to nonspecific toxicity of this pharmacologic agents, we added MG132 to un-transfected

SU-DHL-1 cells. As illustrated in Figure 3-3b, in the absence of SHP1, MG132 induced no increase in the level of NPM-ALK. Thus, the MG132-mediated upregulation of JAK3 and NPM-ALK in SHP1-transfected SU-DHL-1 cells is unlikely a non-specific drug effect. No detectable changes in pSTAT3 and JAK3 were observed. There was a slight decrease in NPM, for which the mechanism is unclear.

**4) SHP1-mediated downregulation of JAK3 was mediated through tyrosine dephosphorylation and increased proteosome degradation**

To provide additional evidence that the SHP1-mediated decrease in pJAK3 is not dependent on that of JAK3, we performed a time-course study using SU-DHL-1 cells transfected with pIRES-EGFP-SHP1, and the results are illustrated in Figure 3-4. The decrease in pJAK3 was detectable as early as 4 hours, at which time the protein expression of the transfected SHP1 became first detectable. Both JAK3 and NPM-ALK did not change their protein levels within the first 10 hours after SHP1 gene transfection was performed.

**5) Co-immunoprecipitation studies**

To further substantiate the functional interaction between SHP1 and NPM-ALK, we performed co-immunoprecipitation experiments. We found that transfected SHP1 in SU-DHL-1 co-immunoprecipitated with NPM-ALK (Figure 3-7). After

immunoprecipitation with an anti-ALK antibody, the membrane was first immunoblotted with anti-ALK (upper panel). The same membrane was then immunoblotted with anti-SHP1. These results showed that SHP1 physically interact with NPM-ALK. We then examined the relationship between JAK3 and NPM-ALK, and between JAK3 and SHP1. As shown in Figure 3-5 (lower panel), JAK3 co-immunoprecipitated with NPM-ALK and SHP1. The overall results indicate that SHP, JAK3 and NPM-ALK physically interact with each other.

**6) Expression of SHP1 induced cell cycle arrest but not apoptosis**

To determine the biological effects of SHP1 in ALK+ALCL cells, cell cycle analysis using flow cytometry was performed. As shown in Figure 3-6, SU-DHL-1 cells transfected with pIRES2-EGFP-SHP1 showed evidence of G1 cell cycle arrest. The proportion of cell in the G0/1 phase was 63%, as opposed to 36% in cells transfected with pIRES2-EGFP. Triplicate experiments were performed and the difference was statistically significant ($p<0.05$, Student t-test). No significant changes in the sub-G0 phase were identified, indicating that there was no substantial increase in apoptosis.

To further assess if the expression of SHP1 induces apoptosis in both cell lines, we performed Western blots using antibodies reactive with PARP and active caspase 3; no evidence of PARP cleaved products or increased active caspase 3

was detected in Karpas 299 and SU-DHL-1 cells transfected with SHP1 (illustrated in Figure 3-7). Nevertheless, SHP1 gene transfection into SU-DHL-1 cells (sorted based on GFP expression) induced a significant decrease in the number of viable cells (assessed by trypan blue exclusion test), with 33% reduction in the number of viable cells 24 hours after gene transfection, and 45% reduction in the number of viable cells 48 hours after gene transfection.

**7) Immunohistochemical studies to compare pSTAT3 and pJAK3 expression in SHP1-positive and SHP1-negative ALK+ALCL tumors**

Previous studies have demonstrated that the vast majority of ALK+ALCL tumors is SHP1-negative; we found that only 14% of ALK+ALCL tumors express the SHP1 protein.[37] Based on our *in-vitro* data presented here, one would predict that SHP1-expressing tumors may have a lower level of activation in JAK3/STAT3 signaling compared to the SHP1-negative tumors. We performed immunohistochemical studies, in which we compared the expression of pSTAT3, pJAK3 and two of the STAT3 downstream targets (Bcl-2 and Mcl-1) in 17 ALK+ALCL tumors (2 SHP1-positive and 15 SHP1-negative). Using >10% pSTAT3 positive cells as the cut-off, we found that 1 of 2 (50%) SHP1-positive tumors was pSTAT3 negative whereas only 1 of 15 (7%) SHP1-negative tumors were pSTAT3-negative. As for pJAK3, 1 of 2 (50%) SHP1-positive tumors was pJAK3 positive, as opposed to 9 of 11 (82%) SHP1-negative tumors assessed. As

for Bcl-2 and Mcl-1, both of which are STAT3 downstream targets that are relatively sensitive to the STAT3 activity *in-vitro*,[27] no significant differences were detected.

## DISCUSSION

SHP1, a tyrosine phosphatase expressed most abundantly in hematopoietic cells, is important in regulating various cellular signaling pathways in lymphocytes.[6] The biological importance of SHP1 is highlighted by the moth-eaten (*me*) mice, in which SHP1 is not expressed (*me/me* phenotype) or dramatically decreased (*meviable/meviable* phenotype). While *me/me* mice die shortly after birth, the homozygous *meviable* mice have patchy hair loss (thus the name 'moth-eaten') as a result of sterile dermal abscesses, abnormal myeloid cell function and development, and an increased propensity of developing CD5+ lymphomas.[39] In humans, gene silencing/methylation of SHP1 has been demonstrated in many hematologic cancers. In view of the normal biological functions of SHP1, loss of SHP1 likely contributes to the pathogenesis of these cancers, although mechanistic studies to define the importance of this biochemical defect are relatively lacking. SHP1 has been previously shown to regulate the JAK/STAT pathway.[5,40,41] Since loss of SHP1 is frequently found in ALK+ALCL,[37] and constitutive activation of JAK3/STAT3 signaling plays an important role in the pathogenesis of ALK+ALCL, we believe that ALK+ALCL is an excellent

experimental model to examine the importance of loss of SHP1 in promoting aberrant JAK3/STAT3 activation and lymphomagenesis.

In this study, we first confirmed that both ALK+ALCL cell lines (Karpas 299 and SU-DHL-1) do not have detectable SHP1 protein expression, and these findings correlated with those of the quantitative RT-PCR assay. The absence of SHP1 expression in SU-DHL-1 is in keeping with that of one of the previously published study,[8] and in keeping with our previous findings that both of these two cell lines have gene methylation of the SHP1 promoter region.[37] In the same experiment, we also documented that SUP-M2, another ALK+ALCL cell line, was SHP1-expressing. This cell line was employed for siRNA silencing of SHP1 expression.

Using gene transfection with SHP1 specific vectors, we successfully transduced SHP1 expression in both Karpas 299 and SU-DHL-1 cells at a relatively high efficiency. Both of these two cell lines have been known to be hard-to-transfect. As far as we know, this is the first study in which relatively high levels of gene transduction using expression vectors were achievable in these cells. We also documented a relatively high rate of siRNA transfection efficiency for SUP-M2 using similar protocol. The availability of these techniques will likely improve our ability to examine the pathogenesis of ALK+ALCL.

In this study, we used two different SHP1 expression vectors for SU-DHL-1 and Karpas 299, since the transfection efficiency appeared to be dependent on the specific cell lines as well as plasmids. While we were able to achieve relatively high levels of SHP1 expression in both cell lines, only the pIRES2-EGFP-SHP1-transfected SU-DHL-1 cells can be sorted by a flow cytometer and purified for analysis. Despite these experimental differences, both cell lines showed similar pattern of results; enforced expression of SHP1 completely abrogated the pJAK3 level in the sorted, SHP1-expressing SU-DHL-1 cells and dramatically decreased the pJAK3 level in the unsorted, SHP1-expressing Karpas 299 cells. These findings are in keeping with the concept that SHP1 serves as a tyrosine phosphatase for JAK3,[1,2,40] thereby negatively regulating JAK3 function. The decrease in pJAK3 was more prominent in SU-DHL-1 cells than in Karpas 299, which can be explained by the fact that the SHP1-transfected Karpas 299 cells were not sorted and thus contaminated with non-transfected cells. Interestingly, the total JAK3 level was also substantially decreased after SHP1 expression in both cell lines. The extent of reduction in JAK3 was relatively small compared to that of pJAK3 in both cell lines, indicating that the decrease in pJAK3 is independent of that of JAK3. To support this concept, results from the time course studies showed that pJAK3 was downregulated much sooner than that of JAK3 after SHP1 transfection. Thus,

downregulation of pJAK3 cannot be explained by the decrease in the JAK3 total protein. Taken together, our results led us to conclude that down-regulation of pJAK3 was due to both SHP1-mediated tyrosine dephosphorylation and SHP1-induced downregulation of the total JAK3 protein.

In addition to JAK3 and pJAK3, SHP1 also effectively down-regulated STAT3 activation in both cell lines. These changes can be partly attributed to the decrease in the protein level and activation of JAK3, a physiologic activator of STAT3. Using pharmacologic inhibitors for JAK3, we have previously demonstrated that JAK3 potentiates STAT3 activation in ALK+ALCL cells.[36] Importantly, NPM-ALK, a potent activator of STAT3,[25] was also decreased by SHP1. Thus, there are at least two mechanisms by which SHP1 can inhibit STAT3 activation: 1) tyrosine dephosphorylation of JAK3, and 2) down-regulation of the protein levels of JAK3 and NPM-ALK. Other mechanisms may be involved.

A recent study by Honorat et al also showed that NPM-ALK is a substrate of SHP1, and SHP1 mediates dephosphorylation of NPM-ALK.[42] We also do not exclude the possibility that SHP1 may directly inactivate STAT3, since it has been reported that these two proteins can physically interact with each other in some cell types.[43]

One of the most important observations of this study is that of SHP1-mediated down-regulation of JAK3 and NPM-ALK in ALK+ALCL via the proteosome degradation pathway. Using MG132, a proteosome inhibitor, we found evidence that SHP1 promotes proteosome-mediated proteolysis of both of these two proteins. We also found that SHP1-induced downregulation of pJAK3 was not reversed by MG132 after the restoration of JAK3 expression, further supporting the concept that tyrosine dephosphorylation of JAK3 by SHP1 can be dissociated with the regulation of the JAK3 protein level. Nevertheless, since MG132 is nonspecific pharmacologic agent, further studies are needed to substantiate these findings and to delineate how SHP1 may regulate the rate of proteosome degradation of JAK3 and NPM-ALK. Our co-immunoprecipitation study showed that SHP1 indeed physically interacts with both JAK3 and NPM-ALK, probably forming a complex. The physical interaction between SHP1 and NPM-ALK also has been shown in the recent study by Honorat et al.[42] In view of the tyrosine phosphatase property of SHP1, it is possible that SHP1 may modulate the interaction between NPM-ALK/JAK3 with proteins in the proteosome pathway by regulating the tyrosine phosphorylation status of these proteins. Of note, a previous study that examined NPM-ALK binding partners using mass spectrometry did not identify SHP1,[44] and we believe that this finding is related to the fact that Karpas 299 is a SHP1-negative cell line.

We also examined if there was any changes to the STAT3 downstream targets in ALK+ALCL revealed in our previous study. With only a partial downregulation of pSTAT3 after SHP1 expression, it is not surprising to observe that only a subset of the examined STAT3 downstream targets were downregulated. There were substantial decreases in Mcl-1, Bcl-2 and cyclin D3, and, to a lesser extent, survivin. This pattern of changes suggests that the expression of various STAT3 downstream targets is dependent on different STAT3 activation levels. Thus, a relatively low level of STAT3 activation is sufficient to activate survivin but not Mcl-1 and cyclin D3. Downregulation of cyclin D3 correlates with the G1 cell cycle arrest. In view of the importance of survivin in anti-apoptosis [45,46], it is possible that the lack of apoptosis in our experimental models is due to the relatively high level of survivin that persists in the presence of SHP1.

Overall, our *in-vitro* findings support the hypothesis that loss of SHP1 significantly contributes to the aberrant activation of JAK3/STAT3 and lymphomagenesis of ALK+ALCL. At the time when this manuscript was revised, Honorat et al reported that NPM-ALK is a substrate of SHP1, and that SHP1-mediated tyrosine dephosphorylation of NPM-ALK leads to decreased cell growth and tumorigenecity.[42] Thus, data from Hororat et al is in parallel with that of our study, supporting that loss of SHP1 plays important pathogenetic roles in ALK+ALCL.

Since SHP1 expression in ALK+ALCL tumors is heterogeneous, the biological importance of SHP1 in these tumors can be further validated, by comparing both clinical and biological parameters in SHP1-expressing and SHP1-negative ALK+ALCL tumors. Our findings from the pSTAT3 and pJAK3 immunostaining in 17 cases of ALK+ALCL tumors are in keeping with the concept that loss of SHP1 may be associated with a higher percentage of pSTAT3-positive/pJAK3-positive cells. Nevertheless, due to the relatively small sample size in this study, future studies including a large number of cases are needed in order to draw definitive conclusion. With regard to the relationship between STAT3 and SHP1, a recent study suggests that STAT3 plays direct roles in contributing to gene methylation and silencing of SHP1 in the ALK+ALCL.[47] Thus, STAT3 activation and loss of SHP1 may in fact form a vicious positive feedback loop, in which STAT3 activation promotes gene silencing of SHP1, and the loss of SHP1 potentiates STAT3 activation. In view of the fact that constitutive activation of STAT3 and loss of SHP1 are relativey common in lymphoid malignancies other than ALK+ALCL, disruption of the STAT3/SHP1 interaction may be a potentially useful therapeutic approach in treating various types of hematologic malignancies.

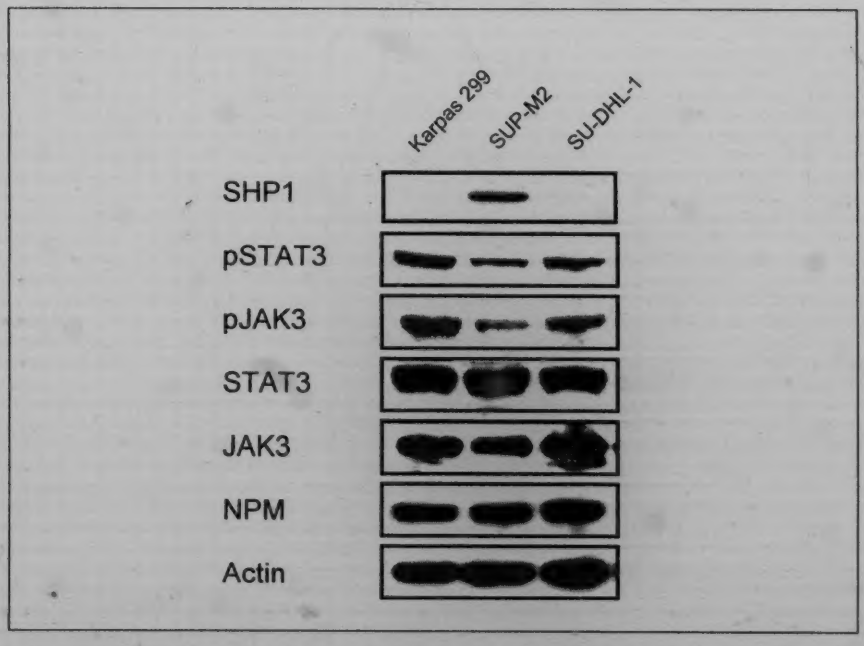


**Figure 3-1 Expression of SHP1, NPM-ALK, JAK3 and STAT3 in three ALK+ALCL cell lines, Karpas 299, SUP-M2 and SU-DHL-1.** Only SUP-M2 expressed SHP1, and this cell line also had relatively lower levels of pSTAT3, pJAK3 and JAK3 compared to the other two cell lines.

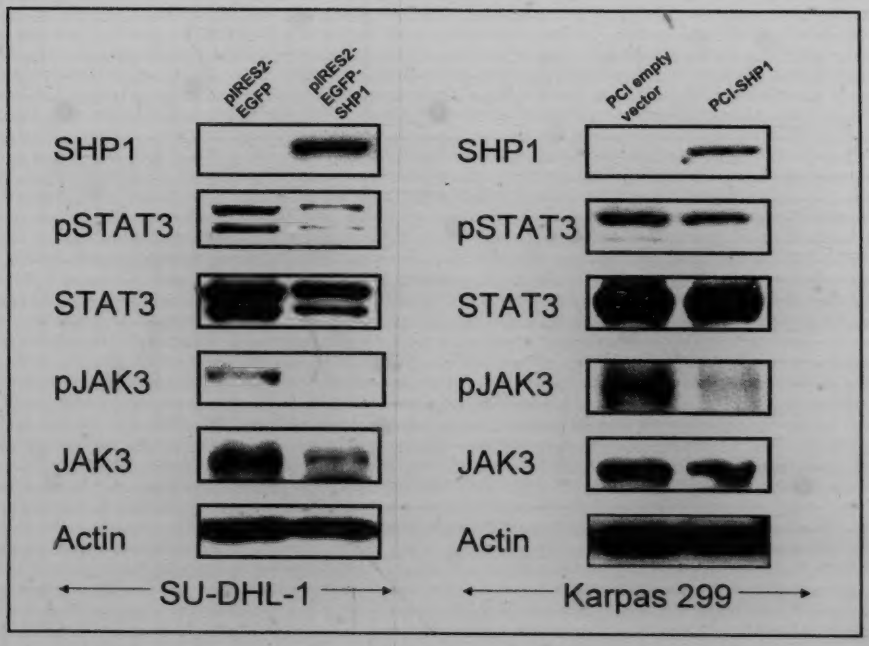


**Figure 3-2a SHP1 expression induced downregulation of the JAK3/STAT3 signaling pathway in SU-DHL-1 and Karpas 299 cells.** SU-DHL1 cells transfected with pIRES2-EGFP or pIRES2-EGFP-SHP1 were sorted using flow cytometry and subjected to Western blot analysis 24 hours after gene transfection. The results showed substantial decreases in the protein expression of pSTAT3, pJAK3 and JAK3. In contrast, there were relatively little changes in the protein level of STAT3 between the two samples. Karpas 299 cells were transfected with pCI(empty vector) and pCI-SHP1, and subjected to Western blot analysis 24 hours after gene transfection. Similar to the SU-DHL-1 cells, Karpas 299 cells showed downregulation of the JAK3/STAT3 signaling,

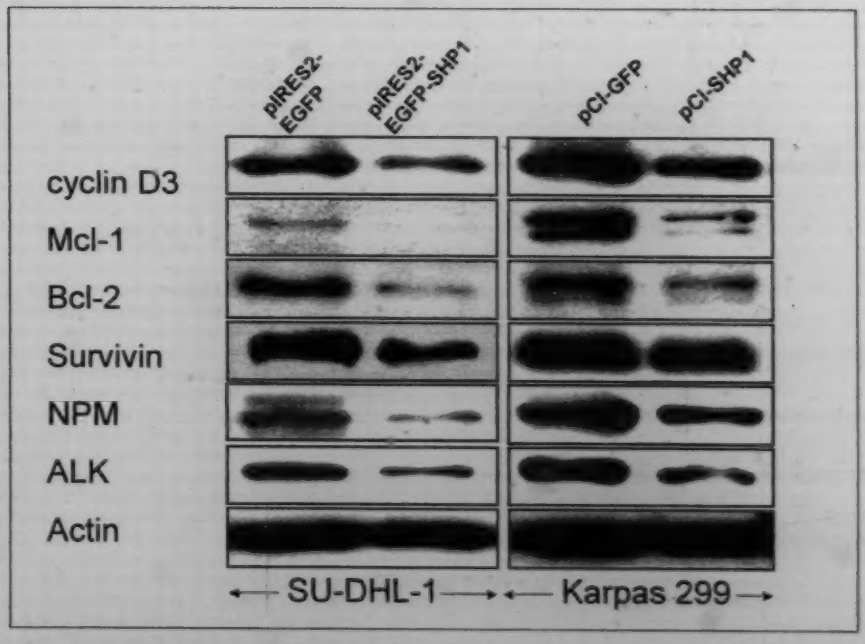


**Figure 3-2b Modulation of STAT3 downstream targets as well as NPM-ALK in Karpas 299 and SU-DHL-1 cells after *SHP1* gene transfection.** SHP1 expression in Karpas 299 and SU-DHL-1 cells induced similar changes in the STAT3 downstream targets including Bcl-2, Mcl-1 and cyclin D3. Survivin was only slightly downregulated. The protein level of NPM-ALK was also decreased. Cell lysates were prepared 24 hours after gene transfection. Cells transfected with pCI empty vector and pIRES2-EGFP served as negative controls for Karpas 299 and SU-DHL-1 cells, respectively.

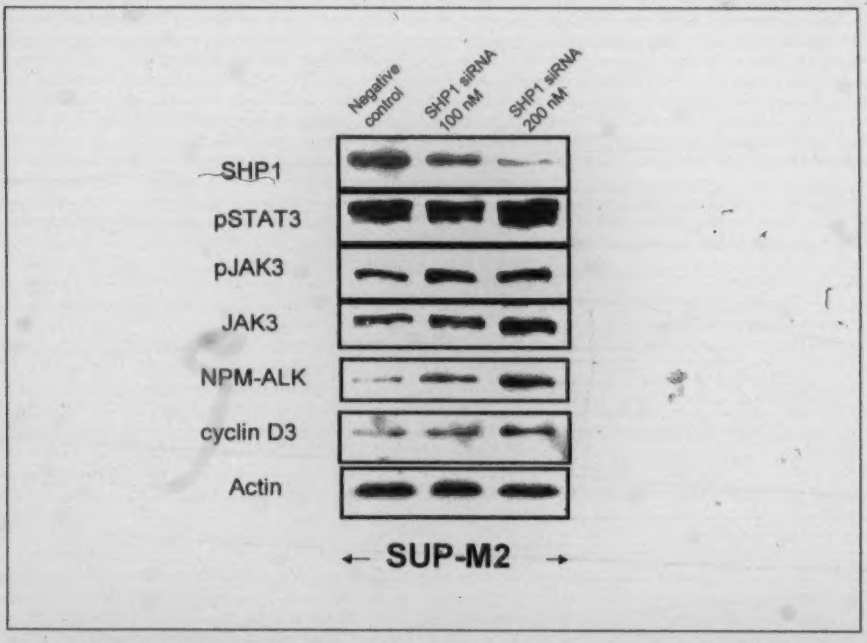


**Figure3-2c Blockade of SHP1 expression using siRNA in SUP-M2.** Inhibition of SHP1 in SU-DHL-1 cells using siRNA induced downregulation of the expression of SHP1, with 200 nM more effective than 100 nM. There were increases in the expression of pSTAT3, pJAK3, JAK3 and NPM-ALK. One of the STAT3 downstream targets, cyclin D3, was also upregulated. SU-DHL-1 cells transfected with the sense SHP1 siRNA species served as negative controls.

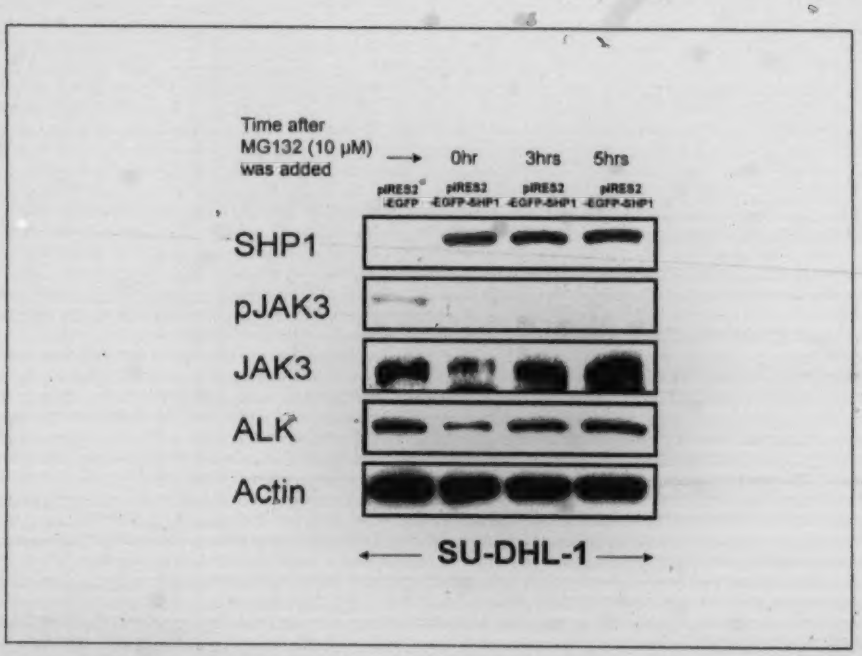


**Figure 3-3a**

**MG-132 reversed the decrease of NPM-ALK and pJAK3 induced by SHP1.** SU-DHL-1 cells were transfected with pIRES2-EGFP-SHP1 and sorted based on GFP expression by flow cytometry. MG132 was then added 24 hours after gene transfection. The reduction in the levels of JAK3 and NPM-ALK induced by SHP1 was completely reversed by MG132 at 3-5 hours after the addition of MG132 to the cell culture. Cells transfected with pIRES2-EGFP (sorted based on GFP expression) served as negative controls.

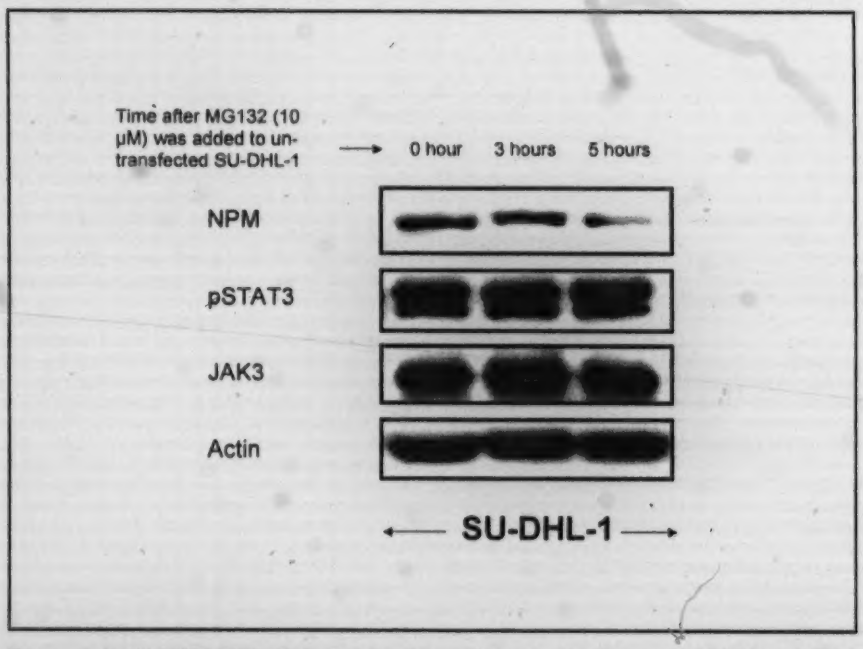


**Figure 3-3b MG132 did not induce upregulation of JAK3, pSTAT3 and NPM-ALK in untransfected SU-DHL-1 cells.** In contrast with the SHP1-transfected SU-DHL-1 cells, un-transfected SU-DHL-1 cells treated with MG132 for 3-5 hours showed no increase in the protein levels of NPM-ALK, pSTAT3, and JAK3.

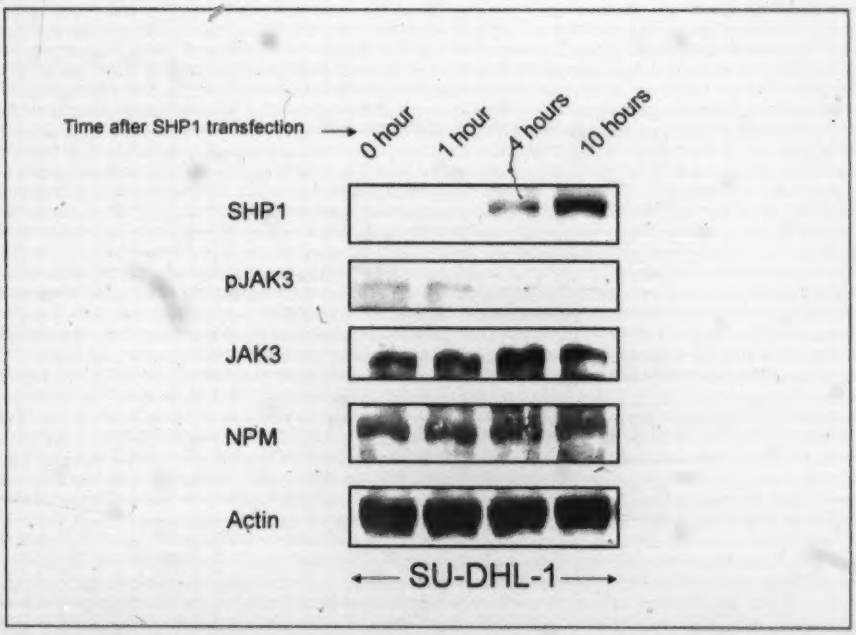


**Figure 3-4 A time course experiment illustrating that SHP1 dephosphorylated JAK3 independent of downregulation of the total JAK3 protein.** SHP1 slowly increased its protein levels within the first 10 hours after SHP1 gene transfection into SU-DHL-1 cells. A decrease in pJAK3 was detectable at 4 hours, at which time no significant changes in the expression of JAK3 and NPM-ALK were observed.

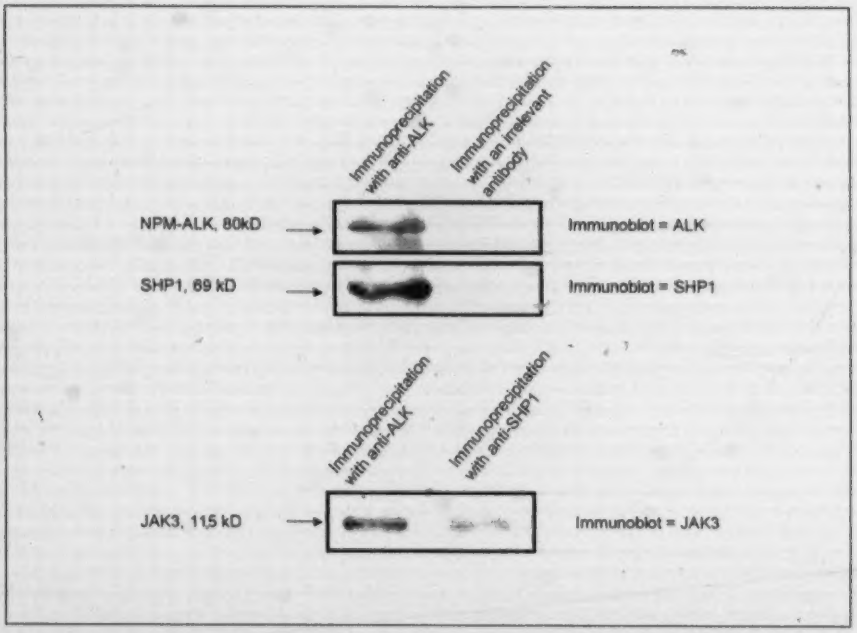


**Figure 3-5 SHP1 co-immunoprecipitated with NPM-ALK and JAK3.** Co-Immunoprecipitation studies indicated that SHP1 physically interacts with NPMALK and JAK3.

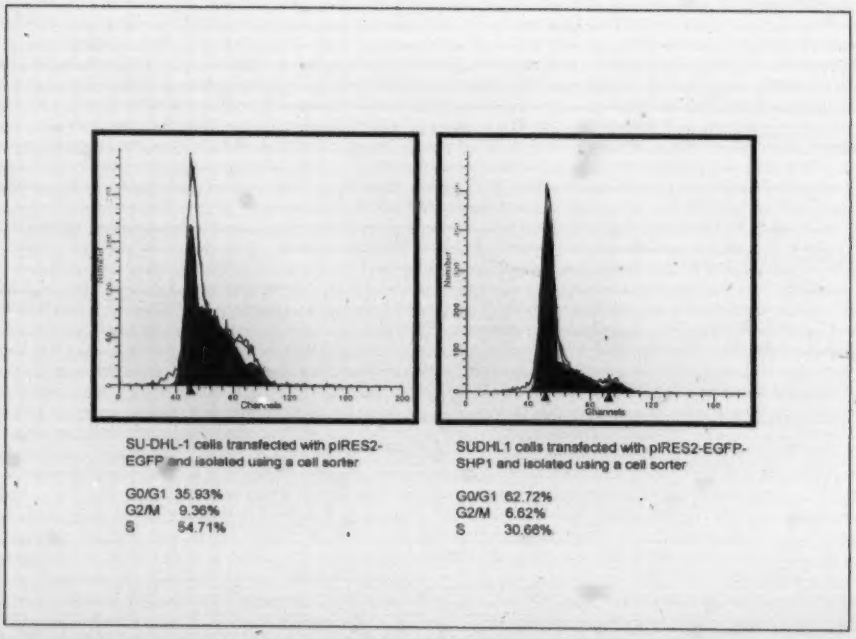
SU-DHL-1 cells transfected with pIRES2-EGFP and isolated using a cell sorter
G0/G1 35.93%
G2/M 9.36%
S 54.71%
SUDHL1 cells transfected with pIRES2-EGFP-SHP1 and isolated using a cell sorter
G0/G1 62.72%
G2/M 6.62%
S 30.66%

**Figure 3-6 SHP1 induced G1 cell cycle arrest and a decrease in the number of viable cells** Cell cycle analysis for SU-DHL-1 cells transfected with pIRES2-EGFP and pIRES2-EGFP-SHP1. Cells were harvested 24 hours after gene transfection and subjected for cell sorting based on GFP expression. All GFP-positive cells were then subjected to cell cycle analysis. SHP1 induced a significant increase in the $G_{0/1}$ population, indicating cell cycle arrest.

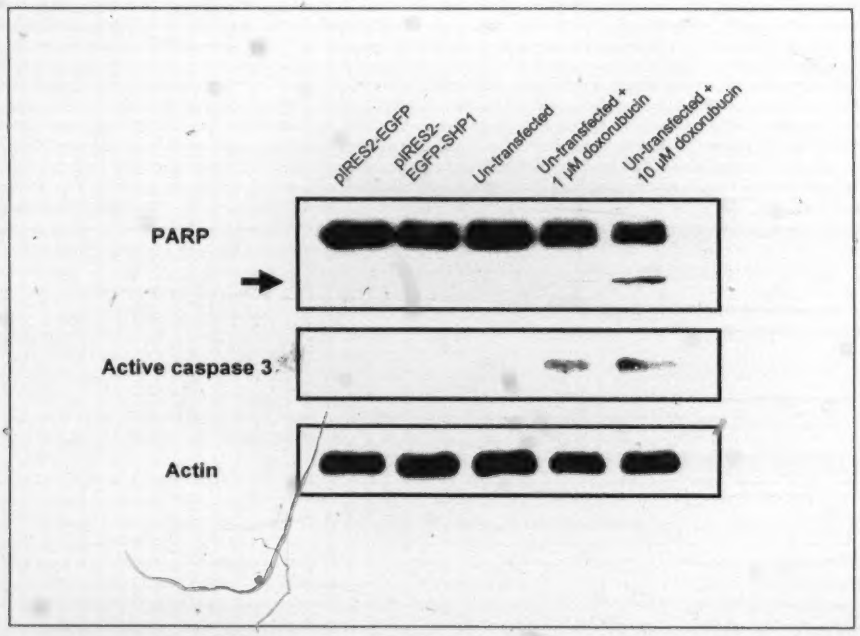


**Figure 3-7 SHP1 induced no significant apoptosis.** In addition to the lack of an increase in the subG$_{0/1}$ cell population, SHP1 expression in SU-DHL-1 cells also did not lead to detectable cleaved PARP product (arrow) and active caspase 3. Untransfected SU-DHL-1 cells treated with different doses of doxorubicin served as positive controls.

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# Chapter 4: General Discussion

## Section 4.1 Introduction to the background

ALK+ ALCL is a type of T/null cell non-Hodgkin lymphoma characterized by the t(2;5)(p23;q35) chromosomal translocation which results in the expression of NPM-ALK, an oncogenic fusion protein. NPM-ALK is a constitutively activated protein tyrosine kinase that can interact with multiple cellular signaling molecules including JAK/STAT3. STAT3 itself is oncogenic,[1] and some oncogenes, such as v-src and NPM-ALK, conduct their transforming effects through STAT3 signaling.[2-4] Constitutively activated STAT3 has been found almost exclusively in ALK+ ALCL tumors and NPM-ALK transformed cells.[3,5,6] As an important suppressor for cytokine signals conducted through the JAK/STAT pathway in hematopoietic cells, SHP1 has been shown to be silent in most ALK+ ALCL because of gene hypermethylation.[7] Considering the normal function of SHP1 in negatively regulating the JAK/STAT3 pathway, we hypothesized that loss of SHP1 expression resulting from promoter hypermethylation contributes to the tumorigenesis in ALK+ ALCL. To test the hypothesis, 5-AZA-2'-deoxycytidine, a pharmaceutic demethylation agent, and SHP1 specific gene transfection, were used to restore SHP1 expression in two SHP1-/ALK+ ALCL cell lines. In addition, we use SHP1-specific SiRNA to effectively decrease SHP1 expression in SHP1+/ALK+ ALCL cell lines and to demonstrate that constitutively activated STAT3 in ALK+ ALCL correlates with the loss of SHP1.

## Section 4.2 Loss of SHP1, due to gene aberrant methylation in ALK+ ALCL, correlates with dysregulated STAT3 activation

We first induced SHP1 expression in SHP1-/ALK+ ALCL by 5-AZA, a DNA methyltransferase inhibitor, and SHP1 mRNA and protein expression were induced successfully in ALK+ ALCL. This result suggests that the promoter hypermethylation is the main reason for the loss of SHP1 in the ALK+ ALCL.

As we expected, the restoration of SHP1 in ALK+ ALCL by 5-AZA correlated with the partly decreased JAK3/STAT3 signaling activity. However, as a drug, the biochemical effects of 5-AZA inside the cells are miscellaneous.[8] To overcome this major technical difficulty, we restored SHP1 expression by gene transfection in Karpas 299 and SU-DHL-1 cells. Though different plasmids were used, we obtained similar results, in which the restoration of SHP1 expression would inhibit JAK3/STAT3 signaling activity in both ALK+ ALCL cell lines. For the SHP1 gene transfection experiment, we are the first to use a plasmid that contained a reporter gene, GFP, which facilitates us to purify the transfected cells.

We further confined the role of SHP1 by downregulating SHP1 expression in SHP1+/ALK+ ALCL and found this correlated with the upregulation of STAT3 activity. By using of siRNA technique, we suppressed SHP1 expression in SUP-M2 cells, which is SHP1+/ALK+. We found that the inhibition of SHP1 would increase JAK3/STAT3 activity and NPM-ALK expression. Therefore, the loss of SHP1 contributes to the dysregulated

STAT3 activity by potentiating STAT3 activation.

## Section 4.3 SHP1 modulates STAT3 activity by downregulating its activators JAK3 and NPM-ALK

We found that SHP1 modulates STAT3 activity by down-regulating its activators' activity. As the main factors that contribute to the aberrant STAT3 activation in the ALK+ ALCL, JAK3 and NPM-ALK are negatively regulated by SHP1 through two mechanisms: (1) inactivating the active form of JAK3/NPM-ALK by dephosphorylation , and (2) accelerating the protein degradation through proteasome pathway.

In both 5-AZA treatment and SHP1 transfection experiments, the restoration of SHP1 is associated with the decrease of pJAK3 and JAK3. To demonstrate that the decrease of pJAK3 is independent to the decrease of JAK3 total protein, we used a time course study and found that the decrease of pJAK3 emerged earlier than the reduction of JAK3. Further, proteasome inhibitor MG132 could restore the decrease of JAK3 expression but not that of pJAK3. These data indicate that SHP1 negatively regulates JAK3 activity by dephosphorylating the active JAK3 and instabilizing JAK3 by accelerating its degradation through proteasome pathway. We have observed in the 5-AZA treatment experiment that pJAK3 did not decrease until two days after SHP1 restoration. Since SHP1 is a rapid response tyrosine phosphatase in cytoplasm, and this is demonstrated in the SHP1 gene transfection experiment that pJAK3 decreased as soon as SHP1 expression (Fig 3-4). We

believe that the lagging in the decrease of pJAK3 after 5-AZA addition is that the initial low level of SHP1 expression induced by 5-AZA is not sufficient in balancing the overactivated JAK3.

One of the key findings of my thesis is the effect that SHP1 exerts on NPM-ALK and negatively modulates its protein stability through the poteasome pathway.

The restoration of SHP1 in both the 5-AZA treatment and SHP1 transfection experiments would decrease NPM-ALK expression. As we known that SHP1 is an SH2 domain containing phosphatase, and this structural characteristic destines that SHP1 could interact with phospho-tyrosyl proteins, such as pJAK3 and NPM-ALK. To test the interaction between SHP1 and NPM-ALK, we performed immunoprecipitation and found that SHP1 physically binds to NPM-ALK and negatively mediates its expression through proteasome pathway. Honorat *et al* (2006)[9] reported that NPM-ALK is a substrate of SHP1 and combined with our result, we can conclude that SHP1 modulates NPM-ALK activity in two methods: dephosphorylating the active NPM-ALK and accelerating the degradation of NPM-ALK through proteasome pathway.

Therefore, SHP1 exerts its effect on STAT3 activity in two ways: inhibits activity and protein stability of JAK3, the physiologic activator of STAT3, and NPM-ALK, the aberrant activator of STAT3. We have demonstrated that JAK3, NPM-ALK and SHP1

physically bind together, and SHP1 could dephosphorylate active JAK3 and NPM-ALK, and accelerate their degradation through proteasome pathway. However, great efforts are needed to elucidate this mechanism.

## Section 4.4 The Vicious feedback between the expression of NPM-ALK and the loss of SHP1

The other key finding of my thesis is that NPM-ALK contributes to silencing of SHP1 in ALK+ ALCL. Previous studies have shown that active STAT3 can form a complex with DNMT1, which binds to SHP1 promoter and facilitates gene methylation of SHP1,[10] Since NPM-ALK is an activator of STAT3,[5] we proposed that the existence of NPM-ALK potentiates the loss of SHP1 in ALK+ ALCL. In support, the results from NPM-ALK gene transfection are entirely in keeping with this concept. As shown in Figure 2-9, SHP1 expression decreased in the NPM-ALK transfected cells compared to the negative controls. This suggests that NPM-ALK contributes to the silencing of SHP1 in ALK+ ALCL by activating STAT3, which could potentiate SHP1 gene methylation. We observed that the forced expression of NPM-ALK in GP-293 cells would not completely eliminate SHP1 expression; therefore, it is likely that additional mechanisms existed to achieve complete SHP1 gene silencing.

The data based on my thesis study to reveal the interaction between JAK3, STAT3, NPM-ALK, and SHP1 as well as their contribution to the pathogenesis of ALK+ ALCL, are

summarized and illustrated as follow (Fig.4-1)

## Section 4.5 Conclusion and the potential future study possibilities

In my thesis study, I have demonstrated that restoration of SHP1 inactivates JAK3 and NPM-ALK and accelerates proteasome degradation of these proteins, and thus restrains STAT3 activity, which leads to cell cycle arrest in G1 and inhibition of cell proliferation. Loss of SHP1 contributes to the dysregulation of STAT3 activity in ALK+ ALCL by enhancing JAK3 activation and stabilizing both JAK3 and NPM-ALK activities. On the other hand, the expression of fusion tyrosine kinase NPM-ALK potentiates the loss of SHP1 expression by activating STAT3, which facilitates SHP1 gene methylation. The point of my thesis is that STAT3 activity is crucial to maintain the dysregulated cell proliferation in ALK+ ALCL, and upregulation of SHP1 expression could be a potential method for the inhibition of aberrant activation of STAT3 signaling in ALK+ ALCL.

It is observed that JAK/STAT signaling is not only mediated by the activity of protein tyrosine phosphatase but also the protein degradation mediated by proteasome.[11,12] Nevertheless, none of these studies explored the mechanisms involved in this process. We observed that both the dephosphorylation and destabilization are involved in the regulating JAK3/STAT3 signaling by SHP1. Interestingly, we observed that STAT3 activity relies on the stabilities of JAK3 and NPM-ALK but not the activity of JAK3. Since MG132 could rescue the degradation of JAK3 and NPM-ALK but not the decrease of pJAK3, and

STAT3 activity increased when MG132 reversed the degradation of JAK3 and NPM-ALK.

It is well understood that SHP1 dephosphorylates proteins at p-tyrosine sites by binding to the substrates through SH2 domain. However, how does SHP1 mediates proteasome degradation, do these proteins bind through SH2 domains for the proteasome degradation? Does SHP1 mediate JAK3/NPM-ALK degradation before their inactivation? All these questions deserve to be studied.

Zhang *et al* (2005) reported that active STAT3 mediates epigenetic silencing of SHP1 in malignant T lymphocytes via binding to DNMT1. The aberrant promoter methylation of CpG-rich areas of promoter regions is the most frequent mechanism of tumor suppressor gene silencing in human tumors.[13] Based on the study of Zhang *et al* (2005), we believe that two possible mechanisms are involved in the oncogenic function of STAT3: (1) upregulating the transcription of genes that promote cell proliferation or downregulate apoptosis, such as cyclin D3, bcl-2; and (2) prohibiting the transcription of cancer suppressor genes, such as SHP1.

Dysregulated STAT3 activity and aberrant methylation of cancer suppressor genes are common characters of human cancers cells. Comparing to the abundant studies focused on the function of STAT3 as an activator for gene transcription, there are few studies that revealed the fact that STAT3 could act as transcription suppressor for cancer suppressor

genes. The contribution of dysregulated STAT3 activity in the aberrant methylation of cancer suppressor genes deserves more future studies.

We observed that restoration of SHP1 could not completely eliminate STAT3 activity, and introduction of NPM-ALK would not clear out SHP1 expression, these data indicate that the dysregulated STAT3 activity and the loss of SHP1 expression are results of multiple factor interactions. My thesis study is the endeavor to elucidate the roles of SHP1 in the dysregulated STAT3 activity as well as its contribution to the pathogenesis of ALK+ ALCL.

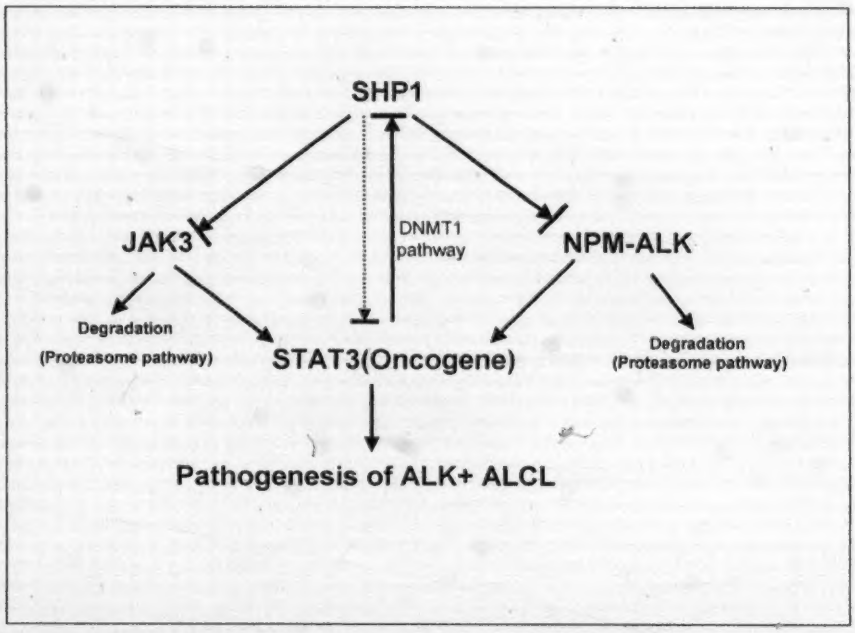


**Fig. 4-1** Schematic diagram to show the interaction between SHP1, JAK3, NPM-ALK and their contribution to the pathogenesis of ALK+ ALCL.

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